A SEROLOGICAL COMPARISON OF BOVINE CORONAVIRUS ISOLATES

By

M. ANN CLARK

Submitted for the degree of Doctor of Philosophy

Moredun Research Institute

and

Edinburgh University

1991

THE UNIVERSITY *of* EDINBURGH

CONTENTS

DECLARATION

I certify that the work presented in this thesis is my own, and that areas of collaboration and assistance from colleagues have been fully identified in the appropriate sections of the text.

M. Ann Clark

M. Ann Clark

October 1991

ACKNOWLEDGEMENTS

I would like to gratefully acknowledge all the people who assisted me in these studies.

I firstly thank my main supervisors, Dr. D. Snodgrass (Moredun Research Institute, MRI) and Dr. M. Norval (Edinburgh University), for providing me with excellent advice and assistance, and for reading this manuscript. I also thank my assistant supervisors, Dr. M. Sharp (MRI) and Dr. M. Ogilvie (Edinburgh University).

I am indebted to many members of staff of the MRI, who contributed both their scientific expertise and their friendship. I would particularly like to acknowledge the help I received from Dr. F. Scott and Dr. G. Browning, from whom I gained a constant wealth of information. I also thank Miss I. Campbell and Mrs. T. Fitzgerald for excellent training and technical support; Mr. E. Dunn and Miss D. Gilfillan for technical assistance; Dr. P. Nettleton and Dr. A. Rae for useful discussions; Mrs. L. Inglis for providing an electron microscopy service; Mrs. K. Hall, Mr. D. McQueen and the staff of the Services and Cell Culture units for a constant supply of equipment and cells; and the staff of the Clinical Department, Animal House and Gnotobiotic Unit for the provision and care of experimental animals.

I also wish to thank those who assisted me in the preparation of this manuscript, particularly Miss J. Goodier for her excellent (and patient) typing; Mr. B. Easter and Mr. A. Inglis for preparing the photographs and for supplying materials from the graphics department; and Mr. M. McLauchlan and Mr. J. Spence for assistance with using the computers.

Finally I thank Coopers Animal Health (latterly Pitman Moore) for funding this project; and Dr. I.D. Aitken, the Director, and Mr. M.J. MacKenzie, the Secretary of the MRI, for allowing me to pursue my research at this Institute.

ABBREVIATIONS

AA	amino acid
Ab	antibody
Ag	antigen
amp	ampere(s)
BCV	bovine coronavirus
BEK cells	bovine embryonic kidney cells
BEL cells	bovine embryonic lung cells
BEV	Berne virus
BFB cells	bovine foetal brain cells
BFS cells	bovine foetal spleen cells
BFTy cells	bovine foetal thyroid cells
BRV	Breda virus
BSA	bovine serum albumin
BUDR	5-bromo-2'deoxyuridine
Bq	becquerel(s)
^{o}C	degrees centigrade
cas-AAs	casamino acids
CCV	canine coronavirus
cDNA	complementary deoxyribonucleic acid
CIP	ciprofloxacine
CK cells	calf kidney cells
cm	centimetre(s)
CO_2	carbon dioxide
CPE	cytopathic effect
C-terminal	carboxy-terminal
Cu	copper
CV	coronavirus
CVL	Central Veterinary Laboratory
D	dalton(s)
DFP	diisopropyl fluorophosphate
DM	dry matter
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DW	distilled water
EBA	epitope blocking assay
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunosorbent assay
EM	electron microscope/electron microscopy
FBK cells	foetal bovine kidney cells
FBS	foetal bovine serum
FCA	Freund's complete adjuvant
FECV	feline enteric coronavirus
FIA	Freund's incomplete adjuvant
Fig.	Figure
FIPV	feline infectious peritonitis virus
FITC	fluorescein isothiocyanate
g	gram(s)
GM	growth medium
gp	glycoprotein
h	hour(s)
HA	haemagglutination
HAI	haemagglutination inhibition

HAT	hypoxanthine-aminopterin-thymidine
HCV	human coronavirus
HCl	hydrochloric acid
HE	haemagglutinin-esterase glycoprotein
HECV	human enteric coronavirus
HEPES	hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HEV	haemagglutinating encephalomyelitis virus
HRP	horseradish peroxidase
HRT-18 cells	human rectal tumour cells
^{125}I	iodine-125
IBV	infectious bronchitis virus
IDUR	5-iodo-2'deoxyuridine
IEM	immune electron microscopy
IF	immunofluorescence
Ig	immunoglobulin(s)
IM	intramuscular
IP	intraperitoneal
IT	infectivity titre
IU	international unit(s)
IV	intravenous
Kb	kilobase(s)
KBq	kilobecquerel(s)
KD	kilodalton(s)
l	litre(s)
log	logarithm (base 10)
M	integral membrane glycoprotein
(figure) M	molar
M strain	Mebus strain
MAb	monoclonal antibody
MBq	megabecquerel(s)
MDBK cells	Madin-Darby bovine kidney cells
MDCK cells	Madin-Darby canine kidney cells
ME	2-mercaptoethanol
MEM	minimal essential medium
$MgCl_2$	magnesium chloride
MHV	mouse hepatitis virus
MI	mock infected
ug	microgram(s)
ul	microlitre(s)
um	micrometre(s)
min	minute(s)
ml	millilitre(s)
mm	millimetre (s)
MM	maintenance medium
moi	multiplicity of infection
MRI	Moredun Research Institute
mRNA	messenger ribonucleic acid
MTM	mixed thymocyte medium
MW	molecular weight
N	nucleocapsid protein
Neu 5, $9Ac_2$	N-acetyl-9-O-acetylneuraminic acid
nm	nanometre(s)
NS	non-structural

N-terminal	amino-terminal
OD	optical density
opd	O-phenylenediamine dihydrochloride
ORF	open reading frame
P	phosphorus
P values	probability values
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS/0.05%T	phosphate buffered saline/0.05% Tween 20
PBS/0.5%T	phosphate buffered saline/0.5% Tween 20
PCV	packed cell volume
PEDV	porcine epidemic diarrhoea virus
PEG	polyethylene glycol
%	percent
PHLS	Public Health Laboratory Service
pi	post infection
PK cells	porcine kidney cells
PMSF	phenylmethylsulphonylfluoride
PRCV	porcine respiratory coronavirus
Pristane	2,6,10,14-tetramethyl-pentadecane
PTA	phosphotungstic acid
R	antigenic relationship
RBC	red blood cell
RbCV	pleural effusion disease virus
RbECV	rabbit enteric coronavirus
RCV	rat coronavirus
RDE	receptor destroying enzyme
RER	rough endoplasmic reticulum
Rf	relative mobility
RIA	radioimmunoassay
RIP	radioimmunoprecipitation
RNA	ribonucleic acid
rpm	revolutions per minute
S	spike glycoprotein
^{35}S	sulphur-35
SDAV	sialodacryoadenitis virus
SDS	sodium dodecyl sulphate
sec	second(s)
SN	serum neutralisation
T	Tween-20
TCA	trichloroacetic acid
$TCID_{50}$	50% tissue culture infective dose
TCV	turkey coronavirus
Temed	N,N,N',N'-tetramethyl-ethylenediamine
TGEV	transmissible gastroenteritis virus
TOC	tracheal organ culture
UK	United Kingdom
USA	United States of America
UV	ultraviolet light
v	volt(s)
VI centre	Veterinary Investigation Centre
VTM	virus transport medium
w	weight
w/w	weight for weight

ABSTRACT

Bovine coronavirus (BCV) is associated with infection of the enteric and respiratory tracts of neonatal calves, and is a cause of neonatal calf diarrhoea. The virus has 4 major structural proteins: the integral membrane glycoproteins (gps) (M), the nucleocapsid proteins (N), the spike gps (S) and the haemagglutinin-esterase gps (HE).

Faecal samples from diarrhoeic calves were tested for BCV using an enzyme linked immunosorbent assay (ELISA), and attempts made to isolate the virus from positive samples in tracheal organ culture (TOC). Growth of the virus was monitored by measurement of haemagglutination (HA) titres, and confirmed by ELISA. Two out of 17 samples (12%) and 29 out of 60 samples (48%) grew to HA titres of at least 16 in TOCs obtained from bovine foetuses and young calves respectively. Seven out of 12 viruses (58%) isolated in calf TOCs were successfully adapted to growth in human rectal tumour (HRT-18) cells (MRI BCV isolates).

Eight monoclonal antibodies (MAbs) were raised against S2 strain BCV (S2 MAbs) and a further 4 MAbs were supplied from the Central Veterinary Laboratory (CVL MAbs). The isotypes of the MAbs were determined, and their protein specificities investigated by Western blotting. One MAb was directed against M, 3 against N, 3 against S and 5 against HE. The MAbs were also characterised in terms of their reactions with S2 virus in immunofluorescence (IF), neutralisation (SN) and haemagglutination inhibition (HAI) tests.

Polyclonal sera and MAbs were used to probe S2 virus proteins in Western blotting experiments. The MWs of the HE, S, N and M proteins were found to be 116 (reducible to 64), 98, 52 and 21 (range 19-23) KD respectively. The S2 MAbs were used in competition ELISAs: the 4 HE MAbs defined a single antigenic region whilst the 3 N MAbs defined 3 distinct regions.

Five standard strains of BCV and the 7 MRI isolates were examined for antigenic variations using polyclonal sera and the S2 MAbs. Polyclonal sera raised against the 5 standard strains failed to detect any strain variations in cross IF and SN tests. Polyclonal anti-S2 serum also failed to detect any strain variations when titrated against the MRI isolates in SN tests and when used to probe the blotted viral proteins of all 12 viruses. S2 MAbs were used in ELISAs, IF, SN and HAI tests, and strain variations were detected on the N, S and HE proteins.

Different species were tested for their potential suitabilities for use as animal models of BCV infection. Forty-three out of 73 mice (59%) developed diarrhoea after oral inoculation with S2 strain BCV, but 27 out of 74 mice (36%) inoculated with mock infected HRT-18 cells also developed diarrhoea. Oral and intranasal inoculation of a gnotobiotic piglet failed to result in diarrhoea, and BCV could not be detected in faecal or nasal swabs. Diarrhoea was successfully produced in a gnotobiotic calf after oral and intranasal inoculation with BCV. Virus was detected in faecal and nasal swab samples, and a strong serological response was observed.

CHAPTER 1

INTRODUCTION

CHAPTER 1
INTRODUCTION
CONTENTS

CHAPTER 1
INTRODUCTION
CORONAVIRIDAE

HISTORY

The first description of a disease caused by a coronavirus (CV) was published in 1931 by Schalk and Hawn. In this paper avian infectious bronchitis was differentiated from other respiratory diseases found in chickens. The causative agent, infectious bronchitis virus (IBV), was isolated in 1937 by Beaudette and Hudson. In subsequent years 2 more coronaviruses were described for the first time. These were mouse hepatitis virus (MHV) (Cheever et al, 1949) and a virus designated B814 which was the first of several new viruses to be isolated from the human respiratory tract (Tyrrell and Bynoe, 1965). In 1968 Almeida et al proposed that these viruses should be grouped together under the name coronavirus. This grouping was largely based on the characteristic surface projections seen when these viruses were examined using an electron microscope (EM). The name coronavirus was chosen because this appearance was reminiscent of both the solar corona and the corona spinarum which surrounds the heads of figures in religious art.

Further work led to a better understanding of the structure and properties of CVs and in 1975 the International Committee on the Taxonomy of Viruses approved the creation of a new family, Coronaviridae, with a single genus, Coronavirus (Tyrrell et al, 1975). The genus originally contained 9 species of virus with IBV as the type species, but several new viruses have been added subsequently.

DISEASES CAUSED BY THE CORONAVIRUSES

Members of the coronavirus family are probably distributed worldwide: they infect many species and cause a variety of clinical diseases. Table 1.1 lists the most commonly recognised CVs and identifies the diseases they cause. Whilst a few CVs (such as MHV and feline infectious peritonitis virus FIPV) affect multiple organs, most CVs are primary pathogens of either the respiratory or enteric tract. Infection of the epithelial cells at these sites results in acute clinical syndromes. Some CVs (IBV, bovine coronavirus BCV, transmissible gastroenteritis virus TGEV, FIPV and MHV) are able to establish persistent infections which are either clinically inapparent or lead to chronic disease syndromes (Underdahl et al, 1974; Wege et al, 1982; Heckert et al, 1990). The existence of persistently infected animals facilitates survival and transmission of the virus and is of economic importance as carrier animals may introduce disease into susceptible populations.

ANTIGENIC GROUPS

The CVs have been divided into 4 antigenic groups: groups I and II infect mammals and groups III and IV infect avians (Table 1.2). Viruses within each group show partial serological cross reactivity. This grouping has been based on results obtained using polyclonal sera and monoclonal antibodies (MAbs) in standard serological tests (Pedersen et al, 1978; Wege et al, 1982; Siddell et al, 1983; Sturman and Holmes, 1983; Holmes, 1990).

Table 1.1

The Coronaviruses: Names, natural hosts and diseases

Virus	Natural host	Diseases
Human coronavirus, HCV*	Man	1. Common cold 2. Lower respiratory disease
Avian infectious bronchitis virus, IBV	Chicken	1. Tracheobronchitis 2. Nephritis 3. Reduction in quantity and quality of eggs
Turkey bluecomb disease virus, TCV	Turkey	Enteritis
Bovine coronavirus, BCV	Cattle	1. Neonatal calf diarrhoea 2. Upper respiratory tract disease 3. Winter dysentery?
Transmissible gastro-enteritis virus, TGEV	Pigs	Gastroenteritis
Porcine respiratory coronavirus, PRCV	Pigs	Respiratory disease (generally subclinical)
Porcine epidemic diarrhoea virus, PEDV	Pigs	Gastroenteritis
Haemagglutinating encephalomyelitis virus, HEV	Pigs	1. Encephalomyelitis 2. Gastroenteritis (vomiting and wasting disease)
Canine coronavirus, CCV	Dog	Gastroenteritis
Feline enteritis coronavirus, FECV	Cat	Enteritis
Feline infectious peritonitis virus, FIPV	Cat	1. Peritonitis, pleuritis 2. Granulomatous lesions
Pleural effusion disease virus, RbCV	Rabbit	1. Pleuritis 2. Myocarditis
Rabbit enteric coronavirus, RbECV	Rabbit	Enteritis
Mouse hepatitis virus, MHV	Mouse	1. Hepatitis 2. Encephalomyelitis 3. Enteritis
Rat coronavirus, RCV	Rat	Rhinotracheitis, pneumonia
Sialodacryoadenitis virus, SDAV	Rat	1. Sialodacryoadenitis 2. Keratoconjunctivitis 3. Rhinotracheitis

*Evidence for the existence of a human enteric coronavirus, HECV, is still inconclusive.

Table 1.2

Antigenic groups of the Coronaviruses

Group 1

HCV-229E and other isolates

TGEV

CCV

FECV

FIPV

Group 2

HCV-OC43 and other isolates

MHV

RCV

SDAV

HEV

BCV

RbCV

Group 3

IBV

Group 4

TCV

Recently published data demonstrates that TCV should be reclassified as a group 2 coronavirus (see page 39).

STRUCTURE

Morphology

CVs particles are pleomorphic to rounded in shape, and have a diameter of 60 to 220 nanometres (nm). The mean diameter of BCV particles is 100 nm, and individual virions vary in size from 90 - 130 nm (Sharpee et al, 1976; Dea et al, 1980b). The buoyant density of CVs in sucrose is about 1.18 grams/centimetre^3 (g/cm^3) (Sturman and Holmes, 1983).

CVs present a characteristic appearance when negatively stained preparations are examined by EM (Fig. 1.1). The virus envelope is seen as a distinct pair of electron dense shells. The spike glycoproteins (gps) radiate from this envelope to form a characteristic fringe of surface projections (spikes or peplomers). The spikes are widely spaced, about 20 nm long and are generally described as being club shaped. Other shapes including rods, cones and mushroom-like projections have also been reported (Davies and Macnaughton, 1979). Virus particles are often seen that either partially or completely lack these surface projections, as they may be lost during virus storage and preparation. Roseto et al (1982) found that a freeze-drying technique resulted in better preservation of BCV particles than that achieved using negative staining.

Virus proteins

Coronaviruses have 3, or less commonly, 4 major structural proteins. These are the nucleocapsid protein N, the integral membrane gp M (formerly matrix gp, E1), the spike gp S (formerly peplomer gp, E2) and the haemagglutinin-esterase gp HE (formerly

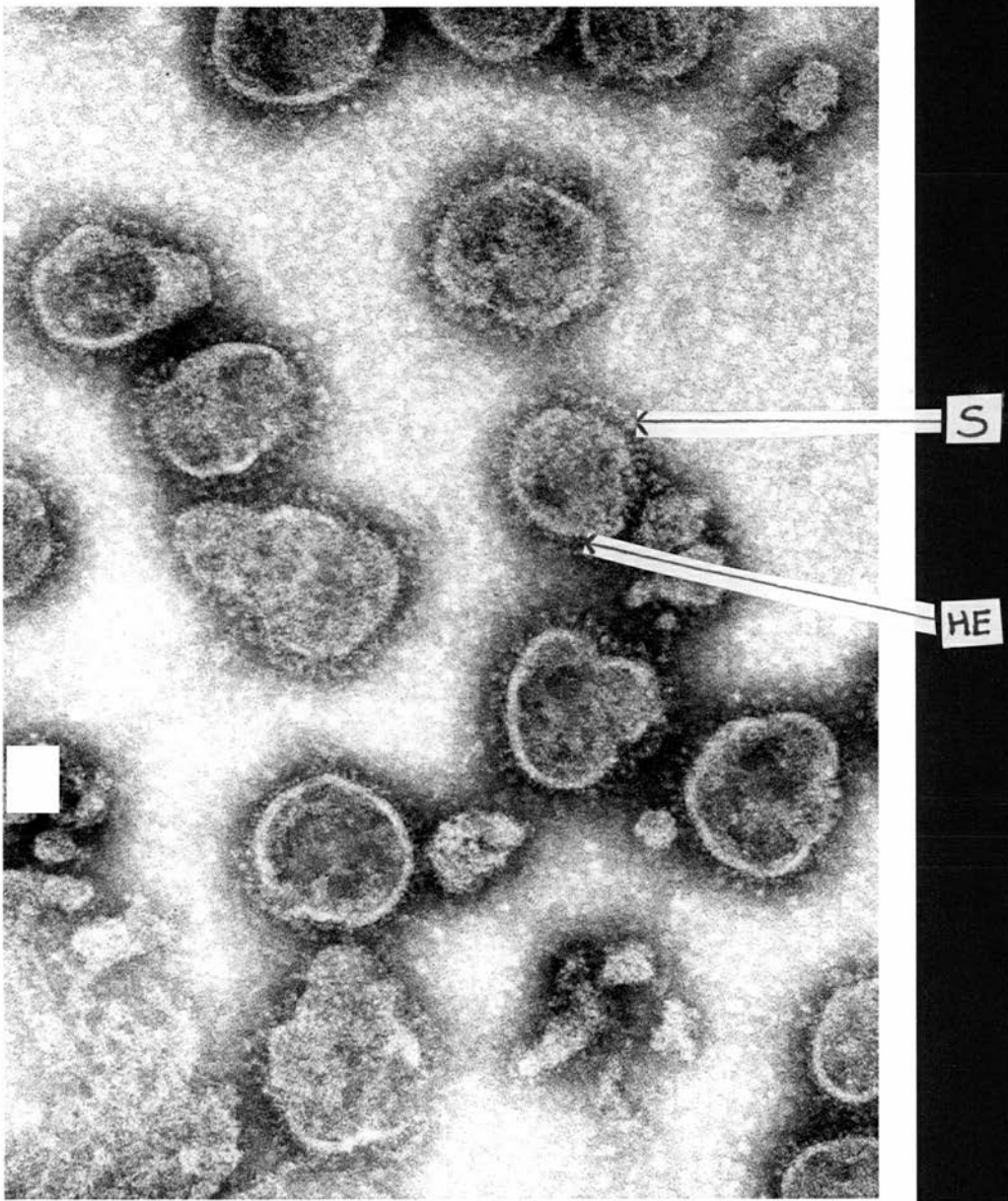
Figure 1.1

BCV particles viewed under the electron microscope

The virus particles in a calf faecal sample were absorbed onto the EM grid using anti-BCV IgG, and negatively stained with phosphotungstic acid.

Total magnification x 160,000.

This photograph was kindly supplied by Mrs. L. Inglis, Moredun Research Institute.



HE haemagglutinin-esterase glycoprotein

S spike glycoprotein

haemagglutinin gp, E3). The N, M and S proteins are found in all CVs whilst the HE gp is present only on some CVs, including BCV.

A model of CV structure is shown in Fig. 1.2 (Holmes, 1990). The N protein is associated with the viral ribonucleic acid (RNA) to form a long, flexible helical nucleocapsid. The M gp spans the viral envelope, whilst the S and HE gps project from the envelope. The HE gp forms the second inner fringe of surface projections which are seen when negatively stained preparations of the viruses possessing this protein are examined by EM (Bridger et al, 1978b). King and Brian (1982) examined the arrangement of the structural proteins of BCV by using pronase enzyme to digest the exposed regions of the virus proteins. These workers found that the enzyme removed the HE, S and M gps to leave digestion resistant fragments with molecular weights (MWs) of 38 and 22 kilodaltons (KD), and that the N protein was left intact.

Nucleocapsid protein, N

The N protein has a MW of 50-60 KD and can form disulphide-linked trimers under non-reducing conditions. This protein is non-glycosylated, but the serine residues are phosphorylated (Siddell et al, 1982). A protein kinase activity has been demonstrated in purified virions which specifically phosphorylates the N protein in vitro. It is unclear whether this enzyme is of viral or cellular origin (Siddell et al, 1981).

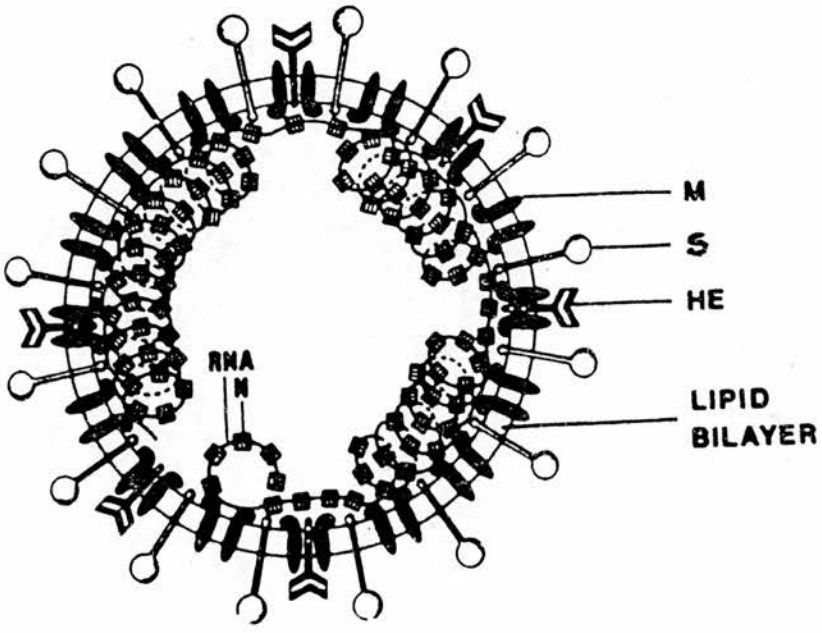
The main function of the N protein is to encapsidate the genomic RNA. Many molecules of the N protein are associated with the RNA to form a long, flexible, helical nucleocapsid, with a diameter of

Figure 1.2.

A diagrammatic model of coronavirus structure

(Adapted from Holmes, 1990).

See text for explanation.


M
S
HE
RNA
N
LIPID
BILAYER

10-20 nm (Sturman and Holmes, 1983). Formation of this nucleocapsid during virus replication facilitates incorporation of the RNA into virion particles.

Integral membrane glycoprotein, M

The M gp exists as a series of species which have different levels of glycosylation on an identical protein backbone. They are seen on protein gels as a series of closely-related bands with MWs in the region of 20-30 KD. High MW bands may also be seen on protein gels as the M gp tends to form aggregates when heated to 100 degrees centigrade (^{o}C) in the presence of sodium dodecyl sulphate (SDS) and 2-mercaptoethanol (ME) (Sturman, 1977).

The M gp is unusual in some CVs such as MHV and BCV because the carbohydrates are joined by O-linked glycosidic bonds (Spaan et al, 1988). This type of bond had not previously been found in viruses. In these CVs the M gp is resistant to the presence of tunicamycin, a compound which inhibits the formation of N-linked glycosidic bonds. In other CVs such as IBV and TGEV, N-linked glycosidic bonds are present (Spaan et al, 1988).

Analysis of sequence data (Armstrong et al, 1984) has led to the proposal that the M gp has 3 distinct domains. A 5 KD hydrophilic amino-terminal (N-terminal) domain lies external to the viral envelope. It contains all the carbohydrate present in the protein and is the only domain which can be removed by pronase or bromelain. The next 80 or so residues form 3 hydrophobic alpha (α) helices which span the lipid bilayer 3 times. The third domain, the carboxy-terminus (C-terminus), is neither strongly hydrophilic

nor hydrophobic. It lies on the inner side of the envelope and interacts with the viral nucleocapsid.

The M gp has several functions. It is structurally important to the virus since it forms part of the virus envelope. During replication it determines the site of virus budding by accumulating at the Golgi and rough endoplasmic reticulum (RER). Here it binds the nucleocapsid to the virus envelope during virus assembly (Sturman et al, 1980; Holmes et al, 1981). Antibodies (Abs) to the M gp have been shown to neutralise virus infectivity in vitro, but this activity may be dependent on the presence of complement (Collins et al, 1982).

Spike glycoprotein, S

The spike gps occur in 2 forms: uncleaved (MW about 200 KD) and cleaved (MW about 100 KD). Cleavage of the primary translation product may be performed by host cell proteases or by trypsin in vitro. The 2 species produced after cleavage may either co-migrate or migrate as separate species on polyacrylamide gels. The N-terminal cleavage product is referred to as S1, the C-terminal product as S2. The extent of cleavage varies with both the virus and the host cell type. In IBV and BCV the protein is mainly cleaved, in HCV only some is cleaved and in TGEV, FIPV and CCV no cleavage occurs. The amount of cleavage in MHV varies from 0 to 100%, depending on the virus strain and the host cell type (Spaan et al, 1988).

Analysis of sequence data reveals further information about the structure of the S gp (Holmes, 1990; Spaan et al, 1988). The

protein is largely hydrophobic and has carbohydrate side chains which are attached by N-linked glycosidic bonds. The C-terminus of the protein is anchored in the lipid bilayer. The rest of the C-terminus (S2) forms a stalk which supports the outer, bulbous N-terminus (S1) domain (Cavanagh and Davis, 1986). In IBV, each spike is made up of a non-covalently linked oligomer of 2 or 3 molecules of the S gp (Cavanagh, 1983). Recent work has demonstrated that the spike of TGEV is a homotrimer of the S polypeptide (Delmas and Laude, 1990).

The S gp has a number of important functions. It binds to the host cell receptor during initiation of infection and is important in membrane fusion events. At the start of infection it induces fusion of the virus envelope with the cell membrane. Later on, it induces cell-to-cell fusion which allows the virus to spread between cells and leads to the formation of syncytia. In some CVs such as MHV, activation of fusion activity requires initial cleavage of the spike gp (Sturman et al, 1985). Other coronaviruses such as TGEV and FIPV do not require cleavage of the S protein for fusion activity. The S gp is an important immunogen as neutralising Abs are directed against this protein (Collins et al, 1982). During virus replication the S gp is transported to the plasma membrane of infected cells, rendering them susceptible to attack by neutralising Abs and cell mediated cytotoxicity (Holmes et al, 1986).

Haemagglutinin-esterase glycoprotein, HE

The HE gp is a disulphide-linked dimer with a MW of 130-140 KD. In the presence of ME it is reduced to two identical monomers, each with a MW of about 65 KD (King et al, 1985). This protein contains N-linked glycosidic bonds.

The HE gp is present in some but not all CVs, and is thought to be involved with haemagglutination. It is found in HCV-OC43, haemagglutinating encephalomyelitis virus (HEV), BCV, turkey coronavirus (TCV) and some strains of MHV (DVIM and JHM) (Spaan et al, 1988). HEV, BCV and TCV are able to haemagglutinate red blood cells (RBCs), whilst some strains of MHV which possess the HE gp are unable to cause haemagglutiantion. Other CVs either totally lack the gene for the HE gp or have an incomplete HE gene which is not expressed. Some of these viruses are totally unable to haemagglutinate RBCs whilst others such as IBV and TGEV cause weak haemagglutination which is mediated by the S gp (Cavanagh et al, 1990). Until very recently it was though that the HE gp was responsible for the ability of the viruses which possessed this gp to cause haemagglutination. It now seems likely however that the S gp in these viruses also plays a role in haemagglutination. At present the precise association of these 2 gps with haemagglutination is unclear: it is possible that only one of these gps is involved or that both gps, acting either independently or together, elicit haemagglutination (Schultze et al, 1991; Storz et al, personal communication).

The HE gp is also thought to allow some CVs to mediate haemadsorption, the process by which RBCs are absorbed to the

membranes of infected cells. In view of recent findings concerning haemagglutination, this action must also be open to question. Two possible mechanisms of haemadsorption have been proposed. The first explanation is that excess HE gp in infected cells is expressed on the cell plasma membrane where it interacts with RBCs directly (Kienzle et al, 1990). The alternative explanation is that virus particles released from infected cells accumulate along the outer surface of the cell membrane, indirectly allowing RBCs to adhere to the infected cell monolayer. This latter phenomenon is termed pseudohaemadsorption (McIntosh, 1974). The HE gp is an important immunogen as Abs to this protein neutralise the virus (Deregt et al, 1983).

Other viral proteins

An additional structural protein (14 KD MW) has been reported for IBV, MHV-A59 and BCV: it is present in very small amounts in the virus envelope. A number of non-structural virus proteins have been demonstrated in infected cells, most notably those having MWs of 14-17 KD, 30-35 KD, and 200 KD. The latter protein is thought to be the viral RNA polymerase enzyme (Babiuk et al, 1985).

Virus envelope: lipids

The virus envelope is derived from the membranes of the Golgi apparatus and RER. The lipid composition of the envelope reflects the lipid content of the host cell membranes (Sturman and Holmes, 1983).

Viral genome

The CV genome consists of a single strand of non-segmented RNA of positive sense. It is 27-30 kilobases (Kb) in size, which is larger than the genome of any other known RNA virus. The genome is polyadenylated at the 3′ end and has a methylated CAP structure at the 5′ end. There is no extensive sequence reiteration.

The genome is organised into 6 or 7 regions, each of which contains 1 or more open reading frames (ORFs). These regions are separated by reinitiation sites (junction sequences) that contain the signal for the transcription of the subgenomic messenger RNAs (mRNAs). The 5′ end of the genome encodes for non-structural viral proteins including the viral RNA polymerase enzyme. The gene order for the structural proteins is 5′-HE-S-M-N-3′ (Spaan et al, 1988).

REPLICATION

A large amount of information is now available on the unique replication strategy of CVs. This work has been summarised in several recent reviews (Siddell, 1987; Spaan et al, 1988; Holmes, 1990; Lai, 1990; Spaan et al, 1990). Replication occurs entirely within the cytoplasm of the cell.

The first stage in replication is the attachment of virus particles to receptors on the target cell membranes. CVs which lack an HE gp bind via their S gp: the host cell receptor for MHV has been recognised as an 110 KD gp (Boyle et al, 1987). The CVs which possess an HE gp may attach to cells by either the S or the HE gp, although a host cell receptor has not been recognised for the S gp of these viruses. The HE gp binds to 9-0-acetylated

neuraminic acid residues on RBC membranes, and it may bind to similar receptors on the host cell plasma membrane (Vlasak et al, 1988b; Schultze et al, 1990). The HE gp also possesses an esterase enzyme which destroys these residues, leading to virus elution from the cell. This enzyme is important in virus replication as the virus grows to a reduced titre if this enzyme is inhibited. It may be involved during endocytosis or virus uncoating (Vlasak et al, 1988a; Vlasak et al, 1988b; Schultze et al, 1990).

The mechanism of virus entry into cells is controversial and may vary between different CVs (Payne et al, 1990a). Virus particles may enter by fusion of the viral envelope with the plasma membrane of the cell. Fusion is mediated by the S gp, and possibly also by the HE gp in CVs which possess this protein (Siddell et al, 1982; Vlasak et al, 1988a). Alternatively entry may be gained by receptor-mediated endocytosis followed by fusion of the viral envelope with the membranes of endocytic vesicles.

Once inside the cell, the genomic RNA attaches to ribosomes and directs the synthesis of RNA dependent RNA polymerase. This enzyme directs transcription of a complementary full length (-) strand of RNA from the genomic (+) strand RNA. The (-) strand RNA serves as a template for synthesis of both full length genomic RNA and also 5 to 7 subgenomic mRNAs. The subgenomic mRNAs have MWs of 0.6×10^6 to 3.7×10^6 KD and form a 3′ nested set with common 3′ ends. Each mRNA contains all the nucleotide sequence of the next smallest mRNA plus an extra gene at the 5′ end which is referred to as the unique region. The 3′ ends of the genomic and subgenomic mRNAs have poly A tails. Their 5′ ends are capped and have a common leader

sequence of 60-70 bases which originates from the 3′ end of the (-) strand RNA.

The location of the leader sequence at the 5′ end of each subgenomic mRNA is thought to result from a discontinuous leader primed transcription of the (-) strand RNA template. The leader RNA transcribed from the 3′ end of the (-) strand RNA separates from the rest of the template but remains bound to the polymerase enzyme. The leader sequence-polymerase complex binds by base pairing at specific non-coding regions called reinitiation sites on the (-) strand RNA. Here it acts as a primer for synthesis of the rest of the mRNA. The degree of complementarity between the leader sequence and the reinitiation site may regulate the expression of the different mRNAs. The mRNAs are synthesised in unequal amounts during infection, but the proportion of each remains constant throughout the replication cycle.

There may be 2 distinct RNA dependent RNA polymerases involved in CV RNA transcription. The first enzyme is active during the early stages of virus replication and directs the synthesis of the (-) strand template. The second enzyme acts later to direct (+) strand synthesis and requires the presence of a primer for transcription.

The CV Study Group recently recommended that the mRNAs should be numbered in order of decreasing size. The genome sized mRNA is therefore referred to as mRNA 1 (Cavanagh et al, 1990). With the exception of the smallest species the mRNAs are polygenic, but only their unique regions are translated. Most unique regions contain a single ORF which codes for a single protein, which, apart from the

S gp, is not subsequently cleaved. Exceptions to this do occur: some unique regions contain more than one ORF which may or may not be translated and a few proteins undergo post-translational cleavage.

Translation of mRNAs coding for the non-structural and N proteins occurs on free ribosomes in the cell cytoplasm. The N protein interacts with newly synthesised genomic RNA by both sequence specific and non-specific binding to form fragile helical nucleocapsids.

The M, S and HE gps are synthesised on ribosomes at the RER. The M gp is unusual in that it is not glycosylated until it reaches the Golgi apparatus. It accumulates at this site and is not transported to the plasma membrane. The S and HE gps are glycosylated at the RER during protein synthesis. They become anchored into the membrane of the RER and are incoporated into virion particles during the process of budding. The carbohydrate side chains of these proteins are modified by cellular enzymes during transport through the Golgi apparatus, and the S gp may also be cleaved at this site. Excess S and HE gps which have not been incorporated into virions are transported to the plasma membrane of the cell.

The site of CV assembly is determined by the restricted intracellular transport of the M gp. Assembly occurs by budding at the membranes of the RER and Golgi apparatus: budding does not occur at the plasma membrane. Strands of nucleocapsid align on the cytoplasmic surface of these membranes due to an interaction with the M gp. Host cell proteins are replaced by viral gps and whole

virions are pinched off and released into the lumen. At these sites the virions are not susceptible to the host's immune system and this may account for the ability of CVs to establish persistent infections in immune animals. These cells will however be susceptible to attack by the host's immune system if virus proteins are expressed on the infected cell surface.

Some CV particles are released by lysis of dying cells. More commonly they are released from intact cells using the normal secretory mechanism of the cell. In this process virions migrate through the Golgi apparatus and become enclosed in smooth walled vesicles. These migrate to the periphery of the cell and fuse with the plasma membrane to release the virus particles. Large numbers of CVs are often seen on the plasma membrane of infected cells. These are viruses which have been absorbed to the plasma membrane after virion release.

STRAIN VARIATION

Some CVs such as MHV and IBV exhibit a high degree of strain variation. This is due to the frequent occurrence of mutation and recombination events. The unusual replication strategy involving multiple subgenomic mRNAs is responsible for the high frequency of recombination seen in the CVs. Recombination may occur between 2 strains of the same CV, 2 different CVs or between a CV and an unrelated virus (Spaan et al, 1988; Snijder et al, 1991).

EVOLUTION

CVs were first thought to be related to orthomyxo- and paramyxoviruses on the basis of similarities in their size and structure (Berry et al, 1964). More detailed study of CVs revealed that they were an entirely separate group of viruses (Holmes, 1985).

Recent work on the HE gp of CVs has revealed similarities to the HE protein of influenza C viruses. The HE gp of BCV has an amino acid (AA) sequence identity of 29.7% with the HA1 subunit of the HE protein of influenza C viruses (Kienzle et al, 1990). The unexpressed gene encoding the HE protein in MHV-A59 also has a 30% AA sequence homology with this protein in influenza C viruses (Luytjes et al, 1988). The HE proteins of CVs and influenza C viruses have similar activities: they cause haemagglutination (although this function in CVs is currently under debate), they bind to receptors containing N-acetyl-9-O-acetylneuraminic acid and they possess a receptor destroying acetylesterase enzyme (Vlasak et al, 1988a; Vlasak et al, 1988b; Schultze et al, 1990). The CVs may have acquired the HE protein by a non-homologous recombination event from the influenza C viruses (Luytjes et al, 1988).

CVs are also related to the recently recognised Toroviridae family, which so far comprises enteric viruses from 3 different species. These viruses are Berne virus (BEV) which was isolated from a diarrhoeic horse, Breda virus (BRV) which has been detected in the faeces of diarrhoeic calves, and human toroviruses which are found in diarrhoeic stools from children and adults. Toroviruses resemble CVs under the EM as they are pleomorphic and bear peplomers, but toroviruses have distinctive sausage-shaped

nucleocapsids. Both groups of viruses contain single strands of positive sense RNA and have similar replication strategies involving 3' coterminal nested sets of mRNAs. Their proteins however have markedly different migration patterns on polyacrylamide gel electrophoresis (PAGE) and they are antigenically distinct (Woode et al, 1982; Horzinek et al, 1984; Weiss and Horzinek, 1987; Snijder et al, 1990; Snijder et al, 1991).

BOVINE CORONAVIRUS, BCV

INTRODUCTION

BCV was first reported in western Nebraska in the spring of 1971. A field experiment was being conducted to assess the efficacy of attenuated rotavirus as an oral vaccine to prevent neonatal calf diarrhoea. Whilst the vaccine was effective in preventing diarrhoea caused by rotavirus, vaccinated calves in several herds developed diarrhoea at 5-21 days old. No rotavirus was detected, but a CV-like agent was revealed by EM examination of semi-purified faeces (Mebus et al, 1972).

Stair et al (1972) established that this agent was a primary pathogen. Gnotobiotic calves were inoculated with bacteria-free filtrates of diarrhoeic faecal material containing the virus. The calves developed diarrhoea and excreted virus in their faeces. Mebus et al (1973a) were the first to isolate the virus and adapted it to grow in foetal bovine kidney (FBK) cells. At this stage the virus was considered to be a CV on the basis of its morphology and

density. Sharpee et al (1976) further characterised the cell culture adapted virus and confirmed this classification.

PHYSICOCHEMICAL PROPERTIES

The physicochemical properties of BCV have been described by Sharpee et al (1976), Sato et al (1977) and Dea et al (1980b).

BCV contains RNA and is therefore insensitive to inhibitors of deoxyribonucleic acid (DNA) metabolism such as 5-iodo-2'-deoxyuridine (IDUR), 5-bromo-2'deoxyuridine (BUDR) and actinomycin D. The virus is surrounded by a lipid envelope and is therefore sensitive to treatment with ether, chloroform and deoxycholate. It is heat labile, particularly at temperatures greater than 45°C, but divalent cations (1 molar (M) magnesium chloride, $MgCl_2$) partially protect the virus against heat. BCV is relatively stable at pH values between 3 and 11 which allows it to survive passage through the stomach of infected animals. Respiratory CVs such as ~~IBV and~~ the human respiratory CVs are acid-labile (Laporte and Bobulesco, 1981a).

BCV agglutinates RBCs from mice and rats and some isolates can also agglutinate RBCs from guinea pigs and hamsters (Dea et al, 1980b). Sato et al (1977) demonstrated that BCV could agglutinate RBCs from chickens but that this reaction was variable and was dependent on the source of cells. BCV is unable to agglutinate RBCs from horses, cattle, goats, sheep, pigs, dogs, cats, rabbits, humans (O), ducks, geese, pigeons or turkeys.

Sharpee et al (1976), Sato et al (1977) and Dea et al (1980b) demonstrated that the haemagglutinating activity of BCV is largely

independent of temperature: virus kept at 50°C for 60 minutes (min) shows a marked drop in infectivity titre (IT) but the haemagglutination (HA) titre is unaffected. HA titres were shown by these workers to be constant when measured at 4, 25 or 37°C, but an incubation temperature of 25°C was preferable since haemagglutination was more rapid and the end points more clearly defined. In contrast to these findings, Van Balken et al (1978) reported that the haemagglutinating activity of BCV was temperature dependent. Haemagglutination occurred at 4°C, but virus eluted from the RBCs when incubated at 37°C. The latter group of workers used faecal and not cell culture adapted viruses and found that haemagglutination was no longer temperature dependent after the faecal viruses had been adapted to growth in tracheal organ culture (TOC). Recently Storz et al (personal communication) reported that cell culture adapated BCV agglutinated chicken RBCs at 4°C, but that the haemagglutination pattern completely disappeared when the temperature was raised to 37°C.

Murine, rat and hamster RBCs adsorb to cell monolayers infected with BCV. The test may be performed at 4, 25 or 37°C, but RBCs attach more firmly at 4°C (Sharpee et al, 1976).

ISOLATION AND GROWTH IN VITRO

BCV is difficult to isolate in cell culture. Some strains such as LY-138 have proved impossible to grow in vitro and are maintained by oral inoculation of calves (Hajer and Storz, 1978).

BCV was first grown in cell culture by Mebus et al (1973a). Virus was isolated in primary FBK cells and successive passages of

virus were grown in consecutively higher passage numbers of FBK cells. This virus was later adapted to grow in bovine embryonic kidney (BEK-1) cells, a continuous cell line (Inaba et al, 1976). Since then BCV has been isolated and grown in a number of cell lines, including primary calf kidney (CK) cells (Bridger et al, 1978b), Vero, Madin-Darby bovine kidney (MDBK) and porcine kidney (PK-15) cells (Dea et al, 1980a), bovine fetal spleen (BFS) cells (Payne and Storz, 1988), human rectal tumour (HRT-18) cells (Laporte et al, 1979; King et al, 1985) and a subline of Madin-Darby canine kidney (MDCK) cells, MDCK 1 (Schultze et al, 1991). Virus growth in early passages is typically without the production of a recognisable cytopathic effect (CPE). Later passages in some cultures such as Vero and MDBK cells produce a marked CPE characterised by vacuolation, shrinkage and detachment of cells leading to complete destruction of the cell monolayer. Syncytia formation is a feature of the CPE in some cell lines.

HRT-18 cells have proved suitable for the growth of the enteric CVs BCV, human enteric coronavirus (HECV) and CCV (Laporte and Bobulesco, 1981a) and TCV (Dea et al, 1989b). HRT-18 cells were established from a human rectal adenocarcinoma and have the properties of differentiated enterocytic cells. Their free surface is covered in microvilli, upon which the receptors for enteropathogenic CVs are thought to be located (Tompkins et al, 1974). BCV has been reported to grow to titres as high as 5×10^7 tissue culture infective $dose_{50}$/ml ($TCID_{50}$/ml) in this cell line.

Trypsin enhances and promotes growth of BCV in some cell lines. Dea et al (1980a) found that trypsin treatment of Vero cell sheets

prior to virus inoculation resulted in a greatly enhanced viral CPE. Presence of trypsin at 10 microgram/millilitre (ug/ml) in maintenance medium increased virus production and CPE in bovine fetal thyroid (BFTy) and bovine fetal brain (BFB) cells (Storz et al, 1981). In addition to merely enhancing virus replication, trypsin treatment permits growth of BCV in some cell lines such as bovine embryonic lung cells (BEL) which are otherwise non-permissive (Toth, 1982). Addition of exogenous trypsin also promotes or enhances the ability of BCV to produce syncytia in cell lines such as BFS (St. Cyr-Coats et al, 1988; Payne and Storz, 1988), BFTy and BFB (Storz et al, 1981).

Some workers have found that BCV produces plaques when inoculated cultures are overlayed with agarose. Plaques become visible to the naked eye after 2-4 days incubation. They have been demonstrated in HRT-18 (Vautherot, 1981), BFTy, BFB (Storz et al, 1981) and BEK-1 cells (Hirano et al, 1985). The presence of trypsin in the overlay medium greatly enhanced plaque formation in BFTy and BFB cells, but was not found to be advantageous in BEK-1 cells.

The problems of BCV isolation in cell culture led some workers to investigate organ cultures as an alternative system for in vitro growth. Organ cultures of tracheas removed from bovine foetuses (late gestation) and newborn calves have proved successful for the isolation and growth of BCVs (Stott et al, 1976; Bridger et al, 1978b; Thomas et al, 1982; McNulty et al, 1984). BCV does not cause ciliostasis of infected tracheas but its growth is generally monitored by the rise in HA titre in the medium bathing the

cultures (Bridger et al, 1978b). Stott et al (1976) were unable to detect any histological changes in infected cultures. Viral antigens were first detected by immunofluorescent (IF) staining in the cytoplasm of the glandular epithelium at 7 days post infection (pi) and in the cytoplasm of the ciliated epithelium at 14 days pi.

Bridger et al, (1978a) adapted a virus isolated in TOC to growth in intestinal organ culture. The cultures originated from the upper small intestine of a bovine foetus. The virus grew to HA titres up to 4 fold higher than those reached in TOC.

SMALL ANIMAL MODELS

BCVs have been adapted to grow in the brains of neonatal suckling mice using the intracerebral route of inoculation. In the first passage suckling mice develop nervous signs at 3-4 days pi and die around day 4-5. Death occurs more rapidly when infected brain emulsions are used to infect mice in subsequent passages (Kaye et al, 1975; Akashi et al, 1981; Gerna, et al 1981). BCV inoculated by the intranasal route can also cause encephalitis in a low proportion of 1 day old mice (Barthold et al, 1990). Akashi et al (1981) found that virus in the third passage level in suckling mouse brain was virulent for 1 day old mice, rats and hamsters when inoculated by the intracerebral or subcutaneous routes. Bengelsdorff et al (1989) vaccinated mice with BCV by the subcutaneous route and measured their serum Ab responses.

In contrast to these findings, Dea et al (1980b) failed to demonstrate virulence of BCV in neonatal mice after intracerebral inoculation. These workers also failed to demonstrate BCV

replication in neonatal mice, hamsters and guinea pigs after intraperitoneal inoculation and in embryonated hen eggs after inoculation into the chorioallantoic cavity.

STRUCTURE

Morphology

The morphology of BCV and its appearance under the EM is similar to that described previously for other CVs.

Virus proteins

The molar ratio of the 4 main structural proteins of BCV is 3 S:9 HE (gp 65): 25N: 25M (King and Brian, 1982). These proteins have been described by several groups, and their reported MWs are summarised in Table 1.3. A wide variation is apparent in the MWs of the S and HE gps. This may be because they were measured using different techniques or it may reflect strain differences. Laporte and Bobulesco (1981b) found that adaptation of a wild strain (WS^-) to growth in cell culture (strain F15) did not alter the MWs of the proteins.

Nucleocapsid protein, N

The gene encoding the N protein has been sequenced in the Mebus (M) strain of BCV (Lapps *et al*, 1987) and in strain F15 (Cruciere and Laporte, 1988): only minor sequence differences were detected between these two viruses. The N protein of BCV is closely related to that of MHV strains A59 and JHM. There is an overall AA sequence homology of 70%, with the areas of closest homology occuring in clusters.

Table 1.3.

MWs of BCV structural proteins

MWs of BCV structural proteins

BCV strain	MW/kD of proteins: S (uncleaved)	S (cleaved)	HE (dimer)	HE (monomer)	N	M	Reference
L-9	180	90		65	50	23	Storz et al, 1981a
M		120/100	140	65	52	26	King and Brian, 1982
M	190	110/90	130	65	52	26	Brian et al, 1983
G110		105/100	125	65	50	26	Vautherot and Laporte, 1983
PQ		100	120	60	50	25,27,28	Deregt et al, 1983
PQ	190	100	124	62	53	23,25,26	Deregt et al, 1987
F15		105/100	125	65	50	28	Cruciere and Laporte, 1988

The N protein gene contains 2 overlapping ORFs. The smallest one potentially encodes a protein of 207 AAs with a MW of 23 KD, but there is no evidence that this protein is expressed. The larger ORF encodes a protein of 448 AAs with a MW of 49 KD: this is the N protein. This protein has 5 regions which are rich in basic AAs and which represent the sites of genomic RNA binding. The N protein is also rich in serine residues, which are clustered in 2 main areas and may be phosphorylated.

Integral membrane glycoprotein, M

The gene encoding the M gp of M strain BCV has been sequenced by Lapps et al (1987). The gene contains a single ORF encoding a slightly basic protein containing 230 AAs with a MW of 26 KD. It has an AA sequence homology of 86% with MHV strain A59. The C-terminal has a net positive charge and may interact with either the negatively charged RNA or with an acidic part of the N protein. The latter interaction was originally proposed by King and Brian (1982) because they found a 1:1 molar ratio of M and N proteins in BCV. The M gp is thought to originate as a 22 KD unglycosylated precursor. Addition of 1 or 2 O-linked oligosaccharide side chains, each of MW about 2 KD, produces species with MWs of 24 and 26 KD. The M gp is therefore seen on PAGE as a series of closely migrating bands (Deregt et al, 1987).

Spike glycoprotein, S

Most authors report that the S protein of BCV is largely present

in its cleaved form (MW about 100 KD), although an uncleaved precursor (MW about 190 KD) is also described (Brian et al, 1983; Deregt et al, 1987). Some cells such as BFS and BFB require the presence of exogenous trypsin to cleave this protein. Cell to cell fusion of these cells only occurs in the presence of exogenous trypsin, which demonstrates that cleavage of the S gp is necessary for activation of its cell fusion activities (Storz et al, 1981b; St. Cyr Coats et al, 1988). Yoo et al (1991) demonstrated that fusion is mediated by the S2 subunit.

The gene encoding the S protein has been sequenced in BCV strains F15 (Boireau et al, 1990), M (Abraham et al, 1990a) and PQ (Parker et al, 1990). A single ORF codes for a protein containing 1363 AAs with a potential MW of 150 KD and 19-20 potential N-linked glycosylation sites. Glycosylation occurs at the RER and Golgi to give an 190 KD gp. Abraham et al (1990) identified a protease cleavage site, permitting cleavage of the S apoprotein into S1 and S2 subunits having MWs of 86 and 65 KD before glycosylation and 120 and 100 KD after glycosylation. A C-terminal hydrophobic alpha helix anchors the S protein in the virus membrane, and two amphipathic alpha helices probably form the stalk of the spike. Comparison with other CVs demonstrates that the BCV S gene is most closely related to the S genes of MHV strains A59 and JHM. The BCV S gene contains a region which is lacking in MHV-A59 and lacking to a greater extent in MHV-JHM. These 3 viruses may therefore have arisen from a common ancestor and evolved either by a series of deletion or recombination events.

The S gp contains important neutralising epitopes. MAbs directed against this protein neutralised the virus both in vitro (Deregt and Babiuk, 1987) and in vivo (Deregt et al, 1989a). Three antigenic regions were recognised in this protein (Deregt and Babiuk, 1987). Regions A and B were defined by strongly neutralising MAbs and analysis of fragments generated by proteolysis of MAb-antigen (Ag) complexes demonstrated that both these regions were situated on a 37 KD fragment (Deregt et al, 1989b). Antigenic region C was defined by a single weakly neutralising MAb. MAbs to antigenic region A lead to in vivo protection, whilst a MAb directed against antigenic region B failed to protect in vivo, possibly due to lack of epitope conservation on the challenge virus isolate (Deregt et al, 1989a).

Haemagglutinin-esterase glycoprotein, HE

The HE gp is a disulphide linked dimer of 2 identical subunits (a homodimer) (King and Brian, 1982; Hogue et al, 1989). The gene encoding this protein has been sequenced in the PQ strain (Parker et al, 1989) and the M strain (Kienzle et al, 1990). The gene contains a large ORF encoding the HE protein and also 2 shorter ORFs which are probably not expressed. The predicted HE protein contains 424 AAs, has a MW of 47 KD and has 9 potential N-linked glycosylation sites. The reported sequences for the HE gp of these 2 strains differ by only 2 bases which lead to a change in just 1 AA.

The primary translation product of the HE gene has been identified as a protein with a MW of 42.5 KD. The 2 subunits are

synthesised independently and then rapidly undergo dimerisation by the formation of disulphide bonds. The protein is then glycosylated and anchored in the membranes of the RER to be incorporated into virion particles during the process of budding. The carbohydrate side chains are processed whilst the virus is transported through the Golgi body (Deregt et al, 1987; Hogue et al, 1989). Not all the HE gp is incorporated into the virion particles: some migrates to the cell surface where it may cause haemadsorption and act as a target for the immune response of the host (Kienzle et al, 1990).

Until recently it was believed that the HE gp was solely responsible for the ability of BCV to mediate haemagglutination. Treatment of the virus with bromelain selectively destroyed the S and M gps, whilst the HE gp and the haemagglutinating properties of the virus remained intact (King et al, 1985). The existence of MAbs to the HE gp which inhibited virus induced haemagglutination supported this hypothesis (Parker et al, 1989). It was found that BCV requires the presence of receptors containing N-acetyl-9-O-acetylneuraminic acid (Neu 5, $9Ac_2$), a sialic acid derivative, on the surface of RBCs in order to cause haemagglutination. Similar receptors are required by the CVs HEV and HCV-OC43 and also by influenza C viruses (Vlasak et al, 1988b; Schultze et al, 1990). Schultze et al (1991) demonstrated that isolated HE gp of BCV bound to these receptors, supporting the proposal that the HE gp is responsible for the haemagglutinating properties of BCV. In contrast to this finding however, the same authors also demonstrated that whilst whole BCV particles agglutinated chicken

RBCs, isolated HE gps were unable to cause haemagglutination. This may suggest that the HE gp is not responsible for causing haemagglutination, in which case HE MAbs which cause haemagglutination inhibition (HAI) must do so either by steric hindrance or by inducing conformational changes in an alternative viral haemagglutinin. It has recently been proposed that the S gp may be involved in haemagglutination, as S MAbs can also cause HAI (Vautherot et al, 1984; Storz et al, personal communication). If the HE gp is indeed the viral haemagglutinin, failure of isolated HE gps to cause haemagglutination may be due to conformational changes occurring during protein purification or may reflect the need for a multivalent attachment of HE gps to RBCs.

A receptor destroying enzyme (RDE) similar to that found in influenza C viruses has been demonstrated in BCV, and is associated with the HE gp (Vlasak et al, 1988a; Vlasak et al, 1988b; Schultze et al, 1991; Storz et al, personal communication). This is a serine esterase (acetylesterase) enzyme which removes the acetyl groups from O-acetyl sialic acids. The enzyme inactivates the cellular receptor for BCV, causing the virus to elute from RBCs at 37^{o}C. Elution may be prevented by inhibition of the RDE with diisopropyl fluorophosphate (DFP) or by MAbs directed against the HE (but not the S) gp (Vlasak et al, 1988a; Storz et al, personal communication). Inhibition of the RDE with DFP also reduces the infectivity of the virus, which suggests that the enzyme is important in early virus replication.

The HE gp is an important immunogen since MAbs directed against this protein neutralise the virus both in vitro (Deregt et al,

1983) and in vivo (Deregt et al, 1989a). A panel of 4 neutralising MAbs defined 4 epitopes on this protein. These were designated epitopes A1, A2, B and C: epitope B overlapped epitopes A and C. Epitopes A1 and A2 were defined by MAbs which had both high serum neutralisation (SN) and HAI titres. Epitopes B and C were defined by MAbs with low SN titres and low or negative HAI titres (Deregt and Babiuk, 1987; Parker et al, 1989). MAbs directed against antigenic region A led to in vivo protection, but a MAb directed against region C failed to protect in vivo (Deregt et al, 1989a).

Non-structural proteins

The genes encoding 5 potential non-structural proteins with MWs of 4.8, 4.9, 9.5, 12.7 and 32 KD have been sequenced (Cox et al, 1989; Abraham et al, 1990b). The gene encoding the 32 KD protein lies immediately upstream of the HE gene. The genes encoding the other 4 proteins lie between the S and M genes. The 9.5 KD protein is expressed on the surface of virus infected cells and may therefore be important in the immune response of the host.

Viral genome

The genome of BCV was first described by Guy and Brian (1979). It has a MW of 6.8×10^6 D (King and Brian, 1982; Lapps and Brian, 1985). The gene map is represented diagrammatically in Fig. 1.3.

STRAIN VARIATION

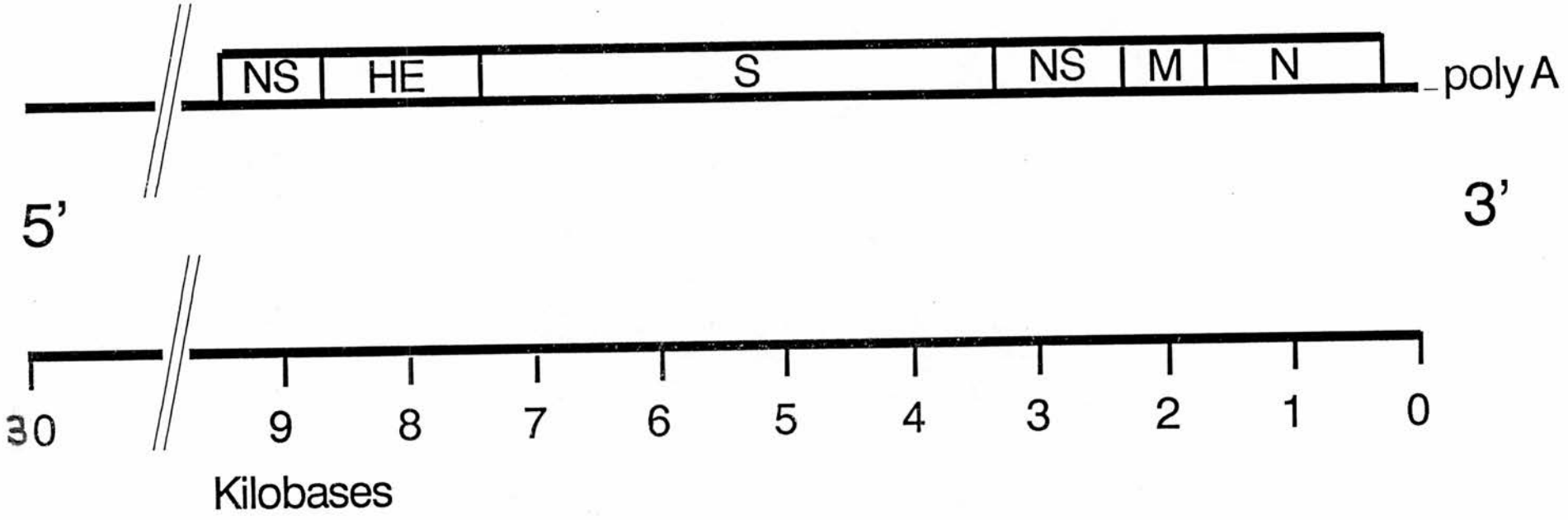
The BCV isolates studied to date all belong to a single serotype. Attempts to find evidence of strain variation have been

Figure 1.3

Gene map of the BCV genome

This diagram has been constructed from data obtained by Lapps et al, 1987; Cruciere and Laporte, 1988; Parker et al, 1989; Abraham et al, 1990a; Abraham et al, 1990b; Boireau et al, 1990; Kienzle et al, 1990 and Parker et al, 1990.

Gene map of the BCV genome



The genome is represented by a horizontal line, running from 5' (left) to 3' (right).

The positions of the sequences encoding the structural proteins (HE, S, M and N) and the predicted non-structural proteins (NS) are shown by rectangles.

limited to examining a small number of isolates due to problems in growing the virus *in vitro*.

Dea *et al* (1980b) demonstrated minor variations in the physicochemical properties of 4 BCVs isolated in Quebec and the M strain of BCV. Antigenic variations amongst these CVs were later detected in counterimmunoelectrophoresis and immunodiffusion tests (Dea *et al*, 1982).

Polyclonal sera have been used in IF, HAI and SN tests to look for evidence of strain variations. Bridger *et al* (1978b) were unable to distinguish 4 isolates by IF and HAI tests. El-Ghorr *et al* (1989) detected strain variations amongst 5 isolates in HAI but not IF and SN tests.

MAbs have detected minor strain variations on the S and HE gps in IF, SN and HAI tests and in enzyme linked immunosorbent assays (ELISAs) (Vautherot and Laporte, 1983; Vautherot *et al*, 1984; Deregt *et al*, 1989a; El-Ghorr *et al*, 1989). Czerny and Eichhorn (1989) used MAbs in a capture ELISA for the detection of BCV in faecal samples. They demonstrated the advantage of using 2 MAbs in place of a single capture MAb to compensate for the effects of strain variation.

Failure to detect major antigenic variation in serological tests is supported by sequence data. The N, S and HE genes have very similar sequences in different virus strains (Lapps *et al*, 1987; Cruciere and Laporte, 1988; Parker *et al*, 1989; Abraham *et al*, 1990a; Boireau *et al*, 1990; Kienzle *et al*, 1990; Parker *et al*, 1990).

ANTIGENIC RELATIONSHIPS

BCV belongs to antigenic group II of the CVs (Table 1.2). Pedersen et al (1978) investigated the antigenic relationships between 8 CVs using polyclonal sera in IF tests. They demonstrated 2-way cross reactions between BCV, HCV-OC43, MHV and HEV. No cross reactions were seen between these CVs and HCV-229E, TGEV, CCV or FIPV. Polyclonal sera detect cross reactions between BCV, HCV-OC43 and HEV in SN and HAI tests and between BCV, HCV-OC43 and MHV strain A59 in Western blotting experiments (Kaye et al, 1975; Sato et al, 1980; Gerna et al, 1981; Brian et al, 1983; Hogue et al, 1984). MAbs raised against BCV cross react in IF tests with HECV, HCV-OC43, MHV and HEV (Vautherot et al, 1983).

The antigenic relationships outlined above are consistent with the results of molecular studies. A 98% genome sequence homology was demonstrated between BCV and HCV-OC43 by T_1 fingerprint analyses (Brian et al, 1983). A cDNA probe to the N and M genes of BCV hybridised with HCV-OC43, HEV and to a lesser extent with MHV in dot blot hybridisation assays (Shockley et al, 1987). Sequence data for the S gp suggests that this protein has arisen from a common precursor in BCV and MHV strains JHM and A59 (Boireau et al, 1990; Parker et al, 1990).

Recent work has demonstrated a close relationship between TCV and BCV. TCV was originally placed in its own antigenic group on the basis of results obtained using immune electron microscopy (IEM) (Ritchie et al, 1973). Since then both polyclonal sera and MAbs have demonstrated close similarities between the structural

proteins of TCV and BCV (Dea et al, 1989a; Dea and Tijssen, 1989b; Dea et al, 1990).

REPLICATION

The precise mechanism by which BCV enters cells is uncertain. Uptake may be achieved either by direct fusion of the virus envelope with the plasma membrane of the cell, or by endocytosis followed by fusion of the virus envelope with the endosomal membranes. It has been proposed that the former mechanism is the major route of virus uptake, and that endocytosis represents an abortive infection as the envelope of endocytosed virions fails to fuse with vesicle membranes and the viruses are subsequently degraded by lysosomal enzymes (Payne and Storz, 1988; Payne et al, 1990a). In contrast to these findings, Yoo et al (1991) proposed that cellular uptake of BCV occurs by endocytosis. The S2 subunit of the S gp is then activated within this acidic intracellular compartment, leading to virus internalisation. This proposal was based on the observation that fusion of insect cells was mediated by the S2 subunit, and that cleavage of the S precursor was necessary for induction of cell fusion.

The morphogenesis of BCV infection has been studied by EM examination of ultra-thin sections of infected intestinal epithelial and tissue culture cells. The information gained from these studies suggests that BCV replicates in a similar manner to the other CVs (Doughri et al, 1976; Tektoff et al, 1983a; Tektoff et al, 1983b). Viral antigens are expressed on the surface of

infected cells, rendering them susceptible to attack by the host's defense mechanisms. Surface expressin of viral antigen may also induce cell to cell fusion in some cell lines (Payne et al, 1990b).

Seven subgenomic mRNAs are produced during BCV replication (Keck et al, 1988). Messenger RNA (mRNA) 3 codes for the HE gp and mRNA 4 codes for the S gp (Parker et al, 1989). Keck et al (1988) demonstrated that there is a differential temporal regulation of synthesis of genomic RNA and the subgenomic mRNAs. During early infection synthesis of subgenomic mRNAs predominates, coinciding with peak virus protein synthesis. Later, synthesis of genomic RNA predominates, coinciding with peak production of infectious virus particles. This suggests that synthesis of genomic RNA may be the rate limiting step in BCV replication.

Messenger RNAs of BCV undergo replication through a (-) strand copy of each mRNA (Hofmann et al, 1990). These (-) strand subgenomic mRNAs form a 5' nested set. A similar situation has been described in TGEV and MHV, and this may provide an important mechanism for amplification of CV mRNA. Since the (+) strand mRNAs (but not the (-) strands) are packaged into completed virions, the possibility exists that these are the only templates required for mRNA replication and that genomic RNA is not therefore necessary for infectivity. These workers also found that mRNA replication continues during persistent infections in cell culture. This suggests that these mRNAs may act in a similar manner to defective interfering RNAs in attenuating virus replication to allow the establishment and maintenance of persistent virus infections.

BCV AND NEONATAL CALF DIARRHOEA

Pathogenesis and pathology

The pathogenesis of BCV in the gastrointestinal tract of the calf has been summarised by Babiuk et al (1985) and the associated physiological changes described by Lewis and Phillips (1978). The resulting pathological findings have been described by Mebus et al (1973b), Mebus et al (1975) and Langpap et al (1979). The precise findings at post mortem depend on the stage of infection at the time of euthanasia or death.

Calves may become infected with BCV by both the oral and respiratory routes. The incubation period is very short as the virus does not have to spread systemically before reaching its prediliction site. Virus infection of the gut starts in the proximal small intestine and spreads throughout the small and large intestines.

Virus replication occurs in the surface epithelial cells, particularly in those on the distal half of the villi in the lower small intestine. Infected cells die, slough off and are replaced by immature cells. Histological examination of the small intestine reveals that the tall columnar epithelial cells of the villi are replaced by cuboidal and squamous epithelial cells at the sides and tips of the villi respectively. Similarly the surface epithelial cells of the large intestine are replaced by low columnar or cuboidal cells, and there is a decrease in the number of goblet cells. Scanning EM reveals marked differences in the length and spacing of the microvilli on individual cells. In the small intestine these changes result in stunting and fusion of adjacent

villi, and there is a large decrease in the ratio of length of villi to depth of crypts. The colonic ridges of the large intestine also atrophy.

The absorptive capacity of the gut is severely diminished by the presence of immature cells and loss of surface area caused by stunting of the villi and atrophy of the colonic ridges. At the same time there is also a decrease in the digestive capacity of the gut, as the immature cells are unable to secrete the normal digestive enzymes. Undigested lactose accumulates in the gut lumen, and the resulting osmotic imbalance draws more water into the gut. The decrease in digestive and absorptive capacities leads to diarrhoea, with loss of water and electrolytes. Gross examination reveals that affected animals are thin and dehydrated, the eyes may be sunken and perineal staining is present. There is also an accumulation of fluid in the small and large intestines.

In severe infections, diarrhoea may lead to dehydration, acidosis and hypoglycaemia, and death may occur due to acute shock and heart failure. More commonly the infection is self limiting, as the virus does not attack the crypt epithelial cells. The mitotic activity of these cells increases, producing immature cells which are more resistant to virus infection and which migrate up the villi to replace the damaged cells. Meanwhile cells including lymphocytes, plasma cells and macrophages infiltrate the lamina propria.

Clinical signs

The severity of BCV enteritis varies with the age and

immunological status of the calf, and the infective dose and possibly the strain of virus. Diarrhoea develops more quickly and is more severe in very young or colostrum deprived calves (Mebus et al, 1973b).

A yellow diarrhoea, possibly containing mucus, develops at about 24 hours (h) pi with BCV. Diarrhoea lasts 3 to 6 days and BCV may be detected in the faeces throughout this period. Calves are often dull and anorexic during the acute stage of infection, and if diarrhoea is severe they may also become pyrexic and dehydrated. The majority of calves recover but a few die if diarrhoea is particularly severe. Discrepancies exist between reports from North America, which suggest that BCV causes a relatively severe diarrhoea, and reports from Great Britain, where BCV strains seem less pathogenic (Mebus et al, 1973b; Bridger et al, 1978b; Crouch et al, 1984; Reynolds et al, 1985; Saif et al, 1986).

Epidemiology

BCV was first reported in western Nebraska, United States of America (USA) (Mebus et al, 1972). Since then it has been reported in many countries including Belgium (Zygraich et al, 1975), Canada (Acres et al, 1975), United Kingdom (UK) and Denmark (Woode et al, 1978), Holland (de Visser et al, 1987) and Switzerland (Battaglia et al, 1986). The virus is probably distributed worldwide. Serological surveys demonstrate that BCV is widespread in cattle populations. Hajer and Storz (1978) detected Ab to BCV in 15 out of 19 herds in South Dakota by an agar gel precipitation test.

Rodak et al (1982) demonstrated the presence of Ab to BCV in 12 out of 12 herds in Saskatchewan using an ELISA test: out of 110 cattle tested, 109 possessed Abs to BCV.

Wide variations occur in the rate of BCV detection in natural outbreaks of diarrhoea. Detection rates vary from 8.5 to 69% in diarrhoeic calves and 0 to 24% in healthy calves (Dea et al, 1979; Reynolds et al, 1986; Snodgrass et al, 1986). Reynolds et al (1986) demonstrated a clear association of BCV with diarrhoea in calves in southern Britain and Wales. BCV was also detected more frequently in diarrhoeic than healthy calves (13% and 4% respectively) in Scotland and northern England, although this difference was not statistically significant (Snodgrass et al, 1986). BCV is often detected in faecal samples from diarrhoeic calves in conjunction with other enteropathogenic agents, particularly rotavirus and also cryptosporidium (Marsolais et al, 1978; Snodgrass et al, 1986).

BCV diarrhoea is more prevalent during the winter months. This may reflect the greater capacity of the virus to survive in a cool, moist environment. It may also result from an increase in the excretion rate of BCV from persistently infected adult cows which occurs during the winter months and at parturition (Collins et al, 1987; Bulgin et al, 1989). Calves in dairy and beef herds are equally affected by BCV enteritis (Langpap et al, 1979; Reynolds et al, 1986). In a survey conducted by Reynolds et al (1986), diarrhoea caused by BCV was found in calves varying in age from 2 to 27 days, with a mean age of 11 days.

Outbreaks of BCV diarrhoea often occur in consecutive years on the same farms. This may be because the virus remains viable in the ground from year to year, but is unlikely as BCV is relatively labile. Outbreaks of diarrhoea still occur, however, if cows are transferred to clean ground at calving. It is possible that clinically normal adult cows which are persistently infected with BCV act as a source of infection for susceptible calves. Chronic shedding of virus can occur from these cows despite the presence of Abs in faeces and serum. In a herd of 121 cows in Saskatchewan, 5% of the cows excreted free virus particles whilst 70% excreted BCV-Ig complexes. The infectivity of the virus in these complexes is unknown (Crouch and Acres, 1984; Crouch et al, 1985). Bulgin et al (1989) found that calves born to carrier animals had a significantly higher risk of developing diarrhoea, and that removal of carrier cows lessened the spread of infection.

Infected calves which persistently excrete virus may also act as a source of infection for susceptible animals (Kapil et al, 1990). Heckert et al (1990) demonstrated that infections with BCV are common in both neonatal and older calves in a closed herd. Infections are often subclinical and may be recurrent in a single animal. Circulation of virus in healthy calves maintains a reservoir of infection for susceptible animals.

Diagnosis: Detection of BCV

BCV infection was originally diagnosed by examination of negatively stained (phosphotungstic acid, PTA) preparations under the EM. Faecal samples may be prepared by sucrose gradient

ultracentrifugation or more simply by microhaematocrit centrifugation (Stair et al, 1972; Durham et al, 1979). Diagnosis by EM is adequate if large numbers of BCV particles are present in the test samples. Virus particles may however be difficult to recognise as they are pleomorphic and tend to lose their peplomers during sample storage and preparation and other fringed particles may also be present in faecal samples. In IEM Abs specific to BCV are used to agglutinate virus particles in order to facilitate their recognition (Langpap et al, 1979; Saif et al, 1986). Protein A gold may be used to highlight these immune complexes (El-Ghorr et al, 1988; Heckert et al, 1989). The latter technique is highly sensitive, but in common with the other EM techniques preparation of the samples is time consuming.

BCV antigen may be detected in intestinal sections using specific polyclonal antisera or MAbs in IF tests. This was used as a diagnostic method in the early work on BCV, but since it necessitates the use of specimens which have been collected from animals within a short time of death it is now only used as a research tool (Mebus et al, 1973b; Langpap et al, 1979; Kapil et al, 1990).

BCV may be detected in faecal samples by virus isolation in either organ cultures of bovine trachea or in cell culture. Virus isolated in organ culture may either be detected in cryostat sections of trachea by IF staining or be detected in the culture medium by EM or measurement of the HA titre (Stott et al, 1976; Bridger et al, 1978b). Virus isolated in cell culture is generally detected by IF staining or haemadsorption, but only the former

method is specific for BCV antigen (Saif et al, 1986; Mostl and Burki, 1989). These techniques are not commonly used for diagnosis of BCV infections, as they are time consuming and very insensitive due to the difficulties of BCV isolation.

Some diagnostic tests are dependent on the ability of BCV to haemagglutinate RBCs. The simplest method is to detect virus in an HA test and use an HAI test to check the positive samples for BCV specificity (Sharpee et al, 1976). The haemadsorption-elution-haemagglutination assay is based on the temperature dependency of CV specific haemagglutinating activity. Virus in faeces is allowed to adsorb to mouse RBCs for 1 h at 4 degrees centigrade (^{o}C). Unadsorbed material is washed off and BCV is eluted from the RBCs by raising the temperature to 37^{o}C. The eluate is then tested in a standard HA test. This method allows the differentiation of BCV specific haemagglutinating activity from non-specific haemagglutinins in the faeces (Van Balken et al, 1978). The reverse passive haemagglutination technique also distinguishes between BCV specific and non-specific haemagglutination. In this technique the test sample is mixed with RBCs coated with BCV specific Abs (Sato et al, 1984). These 3 tests have not been widely used for BCV diagnosis because of problems caused by non-specific haemagglutinins present in faeces and also because different strains of BCV vary in the temperature dependency of their haemagglutination reactions.

ELISAs are the most widely used diagnostic test for BCV. The early tests were based on the use of polyclonal antiserum. Specificity can be improved by incorporation of a positive blocking

stage in which an anti-BCV serum is added after incubation with the faecal samples. A detecting anti-BCV serum is then added which has been raised in a different species to that in which the positive block serum was raised. Depression of the optical density (OD) reading by the positive block serum confirms the specificity of the reaction of the detecting Ab. (Reynolds et al, 1984). More recently MAbs have been prepared to improve the specificity of these assays. They have been used to coat the ELISA plates in an antigen capture system. A mixture of 2 MAbs compensates for BCV strain variations (Czerny and Eichhorn, 1989). The MAbs should ideally be directed against both external and internal viral proteins to allow the detection of both complete and degraded virions (Crouch et al, 1984). Standard ELISAs in which virus is captured by a MAb and detected by an anti-BCV conjugated Ab may fail to detect virus-Ab immune complexes. These are present in faeces in the later stages of clinical disease and during subclinical infections. The problem may be overcome by using an anti-bovine IgG for capture and an anti-BCV conjugated Ab for detection, but this technique will only detect unsaturated immune complexes (Crouch and Acres, 1984).

Dot blot hybridisation assays for detection of BCV RNA have recently been described. These are based on the use of a cDNA probe labelled with either phosphorus-32 (P32) or biotin. At present these techniques are not widely used for the detection of BCV in diagnostic faecal samples (Shockley et al, 1987; Verbeek and Tijssen, 1988; Verbeek et al, 1990; Verbeek and Tijssen, 1990).

Diagnosis: Detection of BCV specific Abs

BCV is very widespread in the cattle population, and the vast majority of adult animals have detectable levels of BCV specific Abs in their serum (Rodak et al, 1982). Measurement of Ab levels in adult animals is therefore only of use when studying outbreaks of winter dysentery (see later). Calves passively acquire protective Abs from their dams in the colostrum, but a rise in the level of BCV specific Abs in paired serum samples is indicative of a recent BCV infection.

The standard range of serological tests may be used to measure BCV specific Ab titres. SN tests are read by plaque reduction, IF staining of virus infected cells or by haemadsorption (Sharpee et al, 1976; Saif, 1987). Serum used in HAI tests must first be treated to remove non-specific agglutinins and non-specific inhibitors of haemagglutination (Sharpee et al, 1976). Passive haemagglutination tests involve the addition of RBCs coated with BCV to dilutions of serum. Non-specific haemagglutination is again a problem in this test, particularly in whey samples (Crouch and Raybould, 1983). ELISAs are widely used to measure the titres of both total Abs and Abs of specific isotypes (Rodak et al, 1982; Crouch et al, 1985; Saif, 1987). BCV Ab titres have also been measured by radioimmunoassay (RIA) (Rodak et al, 1982).

Treatment

The treatment of calves suffering from diarrhoea is aimed at preventing loss of fluids and electrolytes which can potentially lead to dehydration and acidosis. This is achieved by fluid

replacement therapy, which may be accompanied by discontinuing the feeding of milk. The fluids used should be balanced electrolyte solutions and may be given via the oral or intravenous (IV) routes. Oral administration of kaolin mixtures may be used to decrease the severity of diarrhoea. Affected calves should be segregated from healthy animals and kept in a warm area with fresh bedding (Babiuk et al, 1985).

Control

Calves are born agammaglobulinaemic, and initial protection against disease depends on the acquisition of passive immunity from their dams. This is provided by the high levels of Ab present in bovine colostrum at parturition. IgG_1 is the predominant isotype involved, and is largely derived from serum by a selective transport mechanism operating in the bovine mammary gland (Crouch, 1985; Saif, 1985). The level of specific Ab to BCV in the lacteal secretions is very high at parturition but then rapidly declines (Rodak et al, 1982; Crouch and Raybould, 1983).

During the first 24 h of life large amounts of colostral Igs are absorbed from the gut into the systemic circulation. These absorbed Abs provide protection against systemic infections. Protection against enteric infection is dependent on the presence of high levels of Ab in the gut lumen. This is provided by unabsorbed colostral and milk Abs. Duration of passively acquired immunity is prolonged by resecretion of absorbed Abs back into the gut lumen and by adherence of Igs to the surface of the intestinal mucosa (Babiuk et al, 1985; Woode and Bridger, 1975; Moon et al,

1978). Protection against BCV enteric infection correlates better with the local gut Ab titre than the level of Ab in the circulation (Heckert et al, 1990).

Enteric disease occurs when the level of gut Ab falls below that required to protect against the challenge dose of virus. This is most likely to happen if the level of passively acquired Ab declines before an adequate level of active immunity has developed. The offspring of cows, particularly heifers, with low serum Ab titres and therefore low levels of Ab in colostrum and milk are especially susceptible to infection (Woode and Bridger, 1975; Crouch, 1985).

BCV diarrhoea can be prevented either by decreasing the challenge dose of virus to susceptible animals or by increasing their levels of specific immunity.

Decreasing the challenge dose of virus may be achieved through the identification and segregation of carrier cows and calves and general hygienic measures (Radostits and Acres,1980; Crouch and Acres, 1984). This is often impossible in practice as BCV infection may be widespread even in a closed herd (Bulgin et al, 1989; Heckert et al, 1990).

The immune status of susceptible calves to BCV may be raised either by increasing the level of passive protection they obtain from their dams or by stimulating active immunity by oral vaccination. "Calf Guard" (formerly Scourvax II), Norden Laboratories is a vaccine which is commerically available in the USA. It contains attenuated live rota and coronaviruses. It is approved for intramuscular (IM) injection of pregnant cows and oral

inoculation of newborn calves but its efficacy by either route is questionable (Myers and Snodgrass, 1982; Babiuk et al, 1985). Other commercially available BCV vaccines are Coroniffa (Rhone-Merieux) and Lactovac (Hoechst).

Protection against rotavirus diarrhoea in neonatal calves has been achieved by parenteral vaccination of pregnant cows. This raises their circulating IgG_1 levels, increasing the amounts of Ab which are passed to newborn calves in the colostrum. The post parturient decline in colostral Ig levels still occurs, but vaccinated animals continue to secrete higher baseline levels of Ab than non-vaccinates (Snodgrass et al, 1980; Snodgrass et al, 1982). This approach may be used to protect baby mice against infection with BCV (Bengelsdorff, 1988), and a similar technique should be possible in cattle.

Most adult cows have measurable serum Ab titres to BCV as a result of natural infection (Rodak et al, 1982). The aim of vaccination is to stimulate a secondary immune response to increase these Ab levels, raising the levels of protective Abs (mainly IgG1) which are then passively transferred to the suckling calf via the colostrum and milk. The resulting levels of passively acquired immunity in the calf should both prevent disease whilst at the same time permit subclinical infections to stimulate the development of active immunity. If the level of passive immunity was such that infection was totally prevented, then the onset of diarrhoea would merely be delayed until weaning (Saif, 1985). The efficacy of the commercial vaccines presently available for controlling BCV diarrhoea is very questionable (Dauvergne et al, 1983;

Waltner-Toews et al, 1983). This is not surprising, as vaccination of cows with experimental or commercially available vaccines has little or no effect on the levels of BCV specific Abs in the serum, colostrum or milk (Myers and Snodgrass, 1982; Rodak et al, 1982; Dauvergne et al, 1983; Collins et al, 1987). Vaccination may however prevent the increase in shedding of BCV which occurs from carrier cows at the time of parturition (Collins et al, 1987).

Active immunisation by oral inoculation of calves with a live attenuated vaccine against BCV has been investigated. In experimental trials, colostrum deprived calves were protected within 2-3 days of inoculation. In field trials the vaccine was much less effective in preventing BCV diarrhoea (Thurber et al, 1977). This may be due to neutralisation of the vaccine virus by passively acquired maternal Abs. The immune system of the neonate is also immature and may fail to respond adequately to the vaccine (Babiuk et al, 1985; Crouch, 1985).

BCV AND RESPIRATORY TRACT INFECTION

The finding that BCV could be grown in organ cultures of bovine trachea led to the proposal that the virus may also be a respiratory tract pathogen (Stott et al, 1976; Bridger et al, 1978b). This suggestion was supported by the finding that the virus can be grown in vitro in bovine embryonic lung cells (Toth, 1982).

BCV was first reported to be associated with respiratory disease by Thomas et al (1982). Virus was observed in lung wash fluids by EM during a field survey of calf pneumonia outbreaks. The virus

was isolated in TOC and transmitted to gnotobiotic calves. Other workers have also isolated BCV from the respiratory tracts of calves (McNulty et al, 1984), and IF staining of BCV infected nasal epithelial cells has proved to be a very useful method for diagnosing BCV upper respiratory tract infections (Reynolds et al, 1985; Saif et al, 1986). An association between BCV infection and respiratory tract disease has been demonstrated (El-Ghorr, 1988; Mostl and Burki, 1989; Heckert et al, 1990). A significant association has also been found between the simultaneous detection of BCV in faeces and nasal swab samples taken from diarrhoeic calves (Reynolds et al, 1985).

Examination of tissues from experimentally infected calves has demonstrated that BCV attacks the epithelial cells of the nasal cavity and the trachea (Reynolds et al, 1985). Infection may be subclinical or lead to mild upper respiratory signs such as rhinitis, sneezing and coughing. Lower respiratory tract signs are absent, although BCV antigen has occasionally been detected in the lungs and may be associated with minor lung lesions (McNulty et al, 1984; Reynolds et al, 1985). Respiratory infections with BCV are not normally sufficiently severe as to require treatment, although it is possible that they may predispose calves to more severe secondary lower respiratory tract infections (Saif et al, 1986).

BCVs originating from either enteric or respiratory tracts are indistinguishable antigenically. Polyclonal serum and MAbs raised against enteric isolates cross react with respiratory isolates in IEM, ELISAs, HAI and IF tests (Thomas et al, 1982; Vautherot and

Laporte, 1983; McNulty et al, 1984). Antisera raised against viruses isolated from the enteric and respiratory tracts cross neutralise in vitro and cross protect in vivo (Reynolds et al, 1985).

Experimental investigations demonstrate that the outcome of BCV infection is largely unaffected by the origin of the virus (enteric or respiratory) and the route of inoculation (nasal or oral). In all cases infection of the enteric tract results in diarrhoea accompanied by shedding of the virus in the faeces. Infection of the respiratory tract results in virus shedding from the nasal mucosa in the presence or absence of upper respiratory tract signs (Singh et al, 1985; Saif et al, 1986; Saif, 1987). The route of inoculation may affect the time course of disease. Orally infected calves first excrete virus in the faeces whilst intranasally infected calves first excrete virus from the nasal mucosa (Saif et al, 1986).

The epidemiological importance of BCV respiratory tract infections is unclear. Infection can occur in a wide age range of calves and is often subclinical. Clinical signs, when present, are generally seen at about 2-4 months of age (Mostl and Burki, 1989; Heckert et al, 1990). In addition to the faecal-oral route, aerosol infection of calves may be an important route of virus transmission. Spread of virus is assisted by prolonged shedding (up to 2 weeks) of virus from the respiratory tract of infected calves (Saif, 1987; Heckert et al, 1989). BCV is an enveloped virus and is therefore relatively labile. Infection of the respiratory tract may require fewer infectious virus particles than

infection of the enteric tract. Virus multiplication at the former site provides a large number of particles protected by mucus which are then available to seed the enteric tract (Reynolds et al, 1985; Saif et al, 1986).

Immunisation of neonatal calves with live attenuated BCV vaccines given by the respiratory route has been proposed as an alternative method of stimulating active intestinal immunity without the need for primary infection of the gut (Reynolds et al, 1985; Saif et al, 1986). The respiratory route would avoid neutralisation of the vaccine virus by passively acquired Abs present in the gut lumen, but there is still a danger of the virus being neutralised by maternal Abs which have been absorbed systemically. Furthermore, the neonate may fail to respond adequately to the vaccine virus due to immaturity of its immune system.

WINTER DYSENTERY

Winter dysentery occurs during the winter months in housed adult dairy and beef cattle, and is seen most commonly in young adult dairy cows. The disease is characterised by an acute onset of dark, bloody diarrhoea which is accompanied by a dramatic fall in milk production. Animals may also have a nasolacrimal discharge and cough, and may become depressed and anorexic. The disease spreads rapidly within an affected herd (50-100% morbidity) but the mortality rate is very low (1-2%). The clinical disease lasts from a few days to several weeks, depending on the herd size. Milk production losses are very high and production may not return to normal for several months (Saif, 1990).

The causal agent or agents of winter dysentery have still not been fully elucidated, but in recent years BCV has been implicated in some cases. The virus has been detected by EM examination of faeces from affected cattle in New Zealand (Horner et al, 1975; Durham et al, 1979), Japan (Takahashi et al, 1980), France (Espinasse et al, 1982), Belgium (Broes et al, 1984), USA (Saif et al, 1988) and Canada (Durham et al, 1989). The virus has been isolated from diarrhoeic faeces in primary bovine kidney cells (Takahashi et al, 1980) and HRT-18 cells (Broes et al, 1984; Benfield and Saif, 1990), and has been adapted to grow in the brains of suckling mice, rats and hamsters (Akashi et al, 1981). It has yet to be proven whether the virus detected is a causal agent of winter dysentery, an opportunistic invader or part of the normal microflora of the gut. BCV has also been detected in the faeces of normal cows by ELISA and EM, but infectivity of these particles has not been demonstrated (Crouch et al, 1985; Collins et al, 1987). Saif (1990) detected CV particles by IEM in 16 out of 17 diarrhoeic cows and 1 out of 9 healthy cows from herds with outbreaks of winter dysentery. Seroconversion of affected animals to BCV has been demonstrated (Takahashi et al, 1980; Saif, 1990).

The epidemiology of winter dysentery is consistent with BCV being a causative agent. Herds with a prevous history of winter dysentery are more likely to suffer further outbreaks of disease, possibly due to the presence of carrier animals. CVs survive best at low temperatures and at low intensities of ultra violet (uv) light, leading to a higher level of environmental contamination in winter (Pensaert and Callebaut, 1978). Spread of infection may

occur via the oral or respiratory routes, and is facilitated by the close confinement of large numbers of cattle during the winter. TGEV causes similar explosive outbreaks of diarrhoea in pigs during the winter months.

The CV associated with winter dysentery in adult cattle is very closely related to the CV which causes diarrhoea in neonatal calves. Both viruses cause similar pathological changes (Van Kruiningen et al, 1987). A virus which was isolated from a case of winter dysentery (the Kakegawa isolate) is similar in its morphology and physicochemical properties to the M strain of BCV which was isolated from the faeces of a diarrhoeic calf. Neutralisation and HAI tests show that the viruses are serologically closely related (Akashi et al, 1980). In contrast to these findings, Benfield and Saif (1990) found that CVs isolated from cows with winter dysentery in the USA differed from the M strain of BCV in their ability to haemagglutinate RBCs and in cross SN tests.

Attempts have been made to reproduce winter dysentery by oral inoculation of cows with faeces collected from naturally occurring cases of the disease (Kruiningen et al, 1985). These experiments failed to consistently induce diarrhoea in the challenge animals, although CV has been successfully transmitted from adult cows suffering from winter dysentery to gnotobiotic calves (Benfield and Saif, 1990). Reproduction of disease in adult cattle may first require a fuller understanding of the risk factors involved.

These may include the immune status and reproductive state of the animal, environmental stress factors such as changes in diet or low temperatures and the presence of other microorganisms.

ZOONOSIS

It has been reported that BCV may be a zoonotic agent. Storz and Rott (1981) reported the accidental transmission of BCV strain LY-138 from experimentally inoculated calves to a human investigator. Patel et al (1982) reported the replication in a gnotobiotic calf of a CV which had been isolated from the faeces of a human neonate suffering from necrotising enterocolitis.

Neutralising Abs against BCV and HCV-0C43 have frequently been detected in sera from both cattle and man (Gerna et al, 1981; Storz and Rott, 1981; Storz et al, 1981a). This may be due to the zoonotic potential of BCV. A more likely explanation is that cross reactions are occurring between these 2 closely related viruses (Brian et al, 1983; Hogue et al, 1984). The situation is further complicated by the possible existence of a human enteric CV (HECV), which is believed to be even more closely related to BCV than HCV 0C43 (Vautherot and Laporte, 1983).

AIMS OF THIS STUDY

The main aim of this study was to establish the extent of antigenic variation amongst BCV isolates. The possibility of developing a small animal model of BCV infection was also investigated. These studies were divided into the following areas:

1. Isolation and growth of BCVs

Efficient methods for isolating BCVs from faecal samples and for adapting these viruses to growth in cell culture were developed.

2. Production and characterisation of MAbs to BCV

MAbs to BCV were raised and characterised. They were then used to study the viral structural proteins and to investigate the extent of antigenic variation.

3. Analysis of the structural proteins of BCV

The structural proteins of BCV were characterised on a functional basis by studying the reactions of MAbs directed against the different virus proteins in standard serological tests. The MWs of the structural proteins were determined and their epitopes partially mapped.

4. Strain variation of BCVs

The availability of a large panel of BCV isolates permitted a comprehensive investigation of strain variation to be undertaken.

Serological studies were performed with polyclonal sera and MAbs, and the viruses analysed using molecular techniques such as SDS/PAGE and Western blotting.

5. Animal models of BCV infection

The possibility of using conventionally derived neonatal mice, gnotobiotic piglets and gnotobiotic calves as animal models of BCV infection was investigated.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2
MATERIALS AND METHODS
CONTENTS

CHAPTER 2
MATERIALS AND METHODS
MEDIA

RPMI medium: HRT-18 cells

HRT-18 cells were grown in RPMI 1640 medium (Flow Laboratories) supplemented with 0.025M hydroxyethylpiperazine-N'-2-ethane-sulphonic acid (HEPES), 0.008M sodium hydroxide, 0.014M sodium bicarbonate, 0.002M glutamine, 20% heat inactivated foetal bovine serum (FBS), 100 international units (IU)/ml penicillin, 100 ug/ml streptomycin and 2 ug/ml fungizone. This medium is referred to as RPMI growth medium (RPMI GM).

The cells were maintained in the same medium, except that the FBS was reduced to a concentration of 2%. This medium is referred to as RPMI maintenance medium (RPMI MM).

RPMI medium: Hybridoma cells

The hybridoma cells were grown and maintained in RPMI 1640 medium (Flow Laboratories) containing 0.001M sodium pyruvate, 0.002M glutamine, 400 IU/ml penicillin and 400 ug/ml streptomycin. Heat inactivated FBS was included at a concentration of 5, 10 or 15%, the concentration used depending on the state of cell growth.

Methionine free minimal essential medium (MEM): Radiolabelling experiments

Methionine free MEM (1X Eagle's MEM with Earle's salts, Flow Laboratories) was used to maintain HRT-18 cells during virus radiolabelling experiments. It was supplemented with 0.002M

glutamine, 2% heat inactivated FBS, 100 IU/ml penicillin, 100 ug/ml streptomycin and 2 ug/ml fungizone.

199 Medium: TOCs

TOCs were set up in Earle's 199 medium (Flow Laboratories) containing 0.025M HEPES, 0.008M sodium hydroxide, 0.014M sodium bicarbonate, 10% heat inactivated FBS, 0.1% lactalbumin hydrolysate and 0.01% yeast extract. This was supplemented with antibiotics: 200 IU/ml penicillin, 200 ug/ml streptomycin, 4 ug/ml fungizone, 100 IU/ml gentamicin, 50 ug/ml tylosin and 25 ug/ml lincomycin.

The TOCs were maintained in the same medium after virus inoculation, except that the FBS was replaced by 0.5% bovine serum albumin (BSA).

Virus transport medium (VTM)

Nasal swab samples were mixed immediately after collection with VTM, which was supplied by the Service Section at the Moredun Research Institute (MRI). The VTM consisted of Hanks balanced salt solution (Gibco) supplemented with 0.005M sodium bicarbonate, 1% BSA, 0.002% phenol red, 300 IU/ml penicillin, 300 IU/ml streptomycin and 50 IU/ml polymyxin.

CELLS

HRT-18 CELLS

The HRT-18 cells used at the start of these studies had been supplied by the Public Health Laboratory Service (PHLS), Bristol. They were later replaced by cells from PHLS, Birmingham.

Cell culture

Cell culture was mainly performed by the candidate and 2 assistants. Towards the end of these studies the cells were supplied by the Cell Culture Unit at MRI. The cells were grown in 75 cm^2 plastic tissue culture flasks (Flow Laboratories), and passaged at a ratio of 1:4 every 2 or 3 days. When the cells had been passaged about 100 times, new cells were raised from storage in liquid nitrogen.

Mycoplasma testing and decontamination

The HRT-18 cells supplied from Birmingham were known to be contaminated with mycoplasma. These were isolated and identified as Mycoplasma hyorhinis by Dr. A. Rae at MRI. Decontamination was achieved by maintaining the cells in RPMI MM supplemented with 10 ug/ml ciprofloxacine (CIP) for 15 days, during which time the medium was changed every 3 days. The cells were then passaged 3 times without antibiotics, tested again for mycoplasma and found to be negative.

Preparation of HRT-18 cell monolayers for inoculation with virus

Virus stocks were routinely grown in monolayers of HRT-18 cells in 75 cm^2 plastic tissue culture flasks. Viruses were also grown in HRT-18 cells in coverslip cultures. Confluent cell monolayers in flat bottomed 96 well microtitre plates (Nunc) were used for infectivity titrations and SN tests.

Acetone fixation of cells

Virus infected or mock infected (MI) HRT-18 cells were fixed with acetone prior to staining by IF. The medium was removed from the cells, and the cells washed twice with phosphate buffered saline (PBS). Cells in 96 well plates were fixed by adding 50 ul/well of PBS, and then flooding the wells with acetone at 4°C. Coverslip cultures of cells were supported in a rack placed in a bath of acetone. After fixing for 10 min at room temperature, the cells were dried and then either stained immediately or covered and stored at -20°C.

RAT RBCs

Blood was collected into Alsevers solution (0.114M dextrose, 0.03M sodium citrate and 0.07M sodium chloride, pH 6.1) from August or Wistar cross August male rats, and centrifuged at 850g for 5 min at 4°C. The pelleted RBCs were harvested and washed three times with Alsevers solution. They were then resuspended in PBS supplemented with 0.5% BSA (PBS/0.5% BSA) and the packed cell volume (PCV) measured.

RBCs were stored at 4°C for periods of up to 2 weeks for use in HA and HAI tests. The cells were discarded earlier if haemolysis occurred.

TRACHEAL ORGAN CULTURES

The tracheas were obtained from bovine foetuses in late gestation and from neonatal and young Jersey bull calves.

Preparation of the TOCs

The tracheas were removed aseptically and placed in PBS supplemented with 200 IU/ml penicillin, 200 ug/ml streptomycin, 4 ug/ml fungizone, 100 IU/ml gentamicin, 50 ug/ml tylosin and 25 ug/ml lincomycin.

50 millimetre (mm) diameter petri dishes (Sterilin Ltd) were prepared by scoring the bases with a scalpel at appropriate sites for placing the organ cultures. The loose tissues and central connective tissue bands were removed from the tracheas, and they were cut into individual rings. If the trachea had originated from a calf, each whole ring was placed on its side in a petri dish. If the trachea had originated from a foetus, each ring was first cut into 5 pieces and these were placed with their ciliated surfaces uppermost in a petri dish. The cultures were washed 3 times with PBS and 3.0 ml of 199 medium added/dish. They were incubated at 37^{o}C in a 5% CO_2/95% O_2 atmosphere for 1 or 2 days prior to inoculation.

Preparation of the samples

BCVs were isolated in TOC from faecal and nasal swab samples in order to adapt the viruses to growth in vitro and as a method of diagnosing BCV infections.

Faecal samples were prepared by making 1:10 dilutions in PBS containing 100 IU/ml gentamicin and 4 ug/ml fungizone. The suspensions were clarified by centrifugation at 300g for 5 min at 4^{o}C and the supernates collected. Nasal swabs were vortexed

immediately on collection with 1.5 ml of VTM and the resulting suspensions used to inoculate the cultures.

Inoculation of the TOCs

The viability of the organ cultures was confirmed by observing the beating of the cilia using an inverted light microscope (Leitz).

The TOCs were washed 3 times with PBS. 200 ul of the inocula were placed on the epithelial surface of each tracheal ring, 2 rings being inoculated with each sample. The cultures were incubated at 37°C for 1 h in a 5% CO_2/95% O_2 atmosphere. They were then washed twice with PBS, 3.0 ml of 199 media added per dish and the cultures incubated again at 37°C in a 5% CO_2/95% O_2 atmosphere. To prevent cross contamination of viruses between the cultures, each pair of petri dishes was kept in a separate sealed plastic container during all incubation stages. Mock infected negative control cultures were also set up.

The medium bathing the cultures was harvested twice per week and replaced with fresh medium. The HA titres of the harvested fluids were measured and positive samples tested in a diagnostic ELISA for BCV specificity. When isolation in TOC was being used as a diagnostic test for BCV, samples were considered BCV positive if the first or second harvests yielded HA titres of at least 8 and BCV specificity was confirmed by the diagnostic ELISA.

FAECAL SAMPLES

The faecal samples used for virus isolation were sent to the MRI for routine diagnosis (rotavirus and coronavirus) from the Scottish Veterinary Investigation (VI) Centres. They were stored at 4°C prior to testing and BCV positive samples then transferred to -70°C.

BOVINE CORONAVIRUSES

STANDARD STRAINS

S1 and S2 strain viruses had been isolated by Dr. A. El-Ghorr at MRI. They originated from faecal samples collected from 2 field cases of BCV enteritis in Scotland. S1 virus was passaged in 2 gnotobiotic calves (F690 and L965) before being isolated in bovine foetal TOC and adapted to growth in HRT-18 cells. S2 virus was isolated directly from the faecal sample in bovine foetal TOC and adapted to growth in HRT-18 cells (El-Ghorr, 1988).

Three reference strains of BCV had been supplied to MRI from other laboratories and grown in HRT-18 cells by Dr. A. El-Ghorr. The CK strain from England was supplied by Dr. Janice Bridger, Institute for Animal Health, Compton (Reynolds et al, 1985). The Mebus (M) strain, originally isolated in America, was supplied by Dr. S. McNulty, Veterinary Research Laboratory, Belfast (Mebus et al, 1973a). The PQ strain from Canada was supplied by Dr. L. Babiuk, University of Saskatchewan (Dea et al, 1980b).

GROWTH OF BCVs IN HRT-18 CELLS

Growth of BCVs in flasks

BCVs were routinely grown in monolayers of HRT-18 cells in 75 cm^2 tissue culture flasks. The viruses were prepared for inoculation by making a 1:10 dilution in RPMI MM containing 10 ug/ml trypsin and incubated at 37°C for 1h. The cells in each flask were washed twice with PBS, 1.0 ml of inoculum added and the virus allowed to absorb to the cells by incubating at 37°C for 1h. 25.0 ml of RPMI MM containing 1 ug/ml trypsin were then added to each flask, and the flasks incubated at 37°C on a roller for 3 days.

Viruses were harvested by freezing the medium in the flasks at -20°C followed by thawing at 37°C. Stocks were aliquoted and stored at -70°C.

Growth of BCVs in coverslip cultures

BCVs isolated in TOC were initially adapted to HRT-18 cells grown in coverslip cultures. These cultures were inoculated in a manner similar to that described above, except that the virus inoculum was used neat or at a dilution of 1:2, 1:5 or 1:10, and 0.2ml of the virus preparation were inoculated per tube. Some tubes were mock infected (MI) with RPMI MM supplemented with trypsin at 10 ug/ml to act as negative controls. After the virus absorption stage, 1.0 ml of RPMI MM with trypsin at 1 ug/ml was added to each tube. The cultures were then incubated at 37°C for 2 to 3 days on a roller.

The coverslips were examined under an inverted light microscope (Leitz) for the presence of a CPE, and they were then removed for IF staining. Viruses were harvested by freeze/thawing as described above.

Construction of BCV growth curves

Confluent monolayers of HRT-18 cells grown in 50 mm diameter petri dishes (Sterilin Ltd) were infected with S2 strain BCV at an moi of 0.5 or 2.0 (see Virus titrations: Infectivity titres). The procedure used during inoculation was similar to that described previously: 0.5 ml of virus was inoculated per dish and 2.5 ml RPMI MM containing trypsin at 1 ug/ml were added after virus absorption.

Cultures were harvested at appropriate intervals pi by freezing at -20°C for 1 h followed by thawing at 37°C for 20 min (total virus). When using an moi of 0.5, cell-free virus was also harvested by collecting the medium from a second dish and clarifying it by centrifugation at 850g for 10 min at 4°C. The harvests were immediately titrated for infectivity and HA activity and the results obtained used to construct growth curves.

PREPARATION OF STANDARD ANTIGEN COVERSLIPS AND PLATES

Standard antigen coverslips

Coverslip cultures of HRT-18 cells were inoculated in an identical manner to that described above with viruses diluted to ITs of 4.3 log $TCID_{50}$/ml. The cultures were incubated at 37°C for 20 h and then either fixed with acetone before examining the

patterns of IF produced by staining with MAbs, or left unfixed for use in membrane fluorescence tests. MI coverslip cultures were used as negative controls.

Standard antigen plates

The viruses were diluted to ITs of 3.8 log $TCID_{50}$/ml in RPMI MM supplemented with 10 ug/ml trypsin and incubated at 37°C for 1 h. The medium was removed from confluent monolayers of HRT-18 cells in 96 well plates and replaced with 150 ul MM/well. 50 ul of the trypsin activated virus were then added to each well. The plates were covered and sealed, and incubated at 37°C for 2 days. The cells were fixed with acetone, and the standard antigen plates either used immediately or covered and stored at -20°C until required. They were used to screen hybridoma cell supernates for the presence of BCV specific MAbs and to measure the IF titres of polyclonal sera and MAbs. MI antigen plates were used as negative controls.

CONCENTRATION AND PURIFICATION OF BCVs

Concentration of BCVs by ultracentrifugation

Virus infected cell culture harvests were clarified by centrifugation at 1500g for 10 min at 4°C. 5.0 ml of a 10% (weight/weight, w/w) solution of sucrose in TNE buffer (0.01M tris - hydrochloride, 0.1M NaCl, 1.0mM EDTA, adjusted to a pH of 7.5). were pipetted into 35.0 ml plastic ultracentrifuge tubes and the clarified supernates gently layered on top. The tubes were centrifuged in an SW28 rotor on a Beckman L2 or L5 ultracentrifuge

at 100,000g for 45 min. Pelleted viruses were resuspended in appropriate volumes of TNE buffer to give an 100 X concentration by volume of the original viruses. Virus pellets were used immediately or stored at -70°C.

Sucrose gradient purification of BCVs

Viruses which had been concentrated by ultracentrifugation through a sucrose cushion were layered onto 20-60% (w/w) sucrose gradients and centrifuged at 4°C overnight at 52,000g in an SW40 ti rotor on a Beckman L2 or L5 ultracentrifuge. Fractions were collected by piercing the base of the tubes with a needle. The sucrose densities of the fractions were measured on a refractometer and the fractions titrated in HA tests. The fractions which had peak HA titres with sucrose densities of 1.18 ± 0.02 g/cm^3 were pooled, and the viruses concentrated again by pelleting through a sucrose cushion.

POLYCLONAL SERA

REFERENCE ANTISERA SUPPLIED BY MRI

A number of reference polyclonal sera and MAbs which had been produced by the enteritis section, MRI, were used throughout these studies. They are listed in Table 2.1.

REFERENCE ANTISERA RAISED BY THE CANDIDATE

Some reference antisera were also raised by the candidate. These are listed in Table 2.2 and their production is described below.

Table 2.1

Reference antisera and MAbs

(Raised by members of the enteritis section, MRI)

Reference antisera and MAbs*

Serum number	Description	Abbreviated name
1776	Rabbit hyperimmune anti - rotavirus serum	Rabbit anti - rotavirus serum
3626	Gnotobiotic lamb hyperimmune anti - rotavirus serum	Lamb anti - rotavirus serum
4381	Rabbit hyperimmune anti - rotavirus serum	Rabbit anti - rotavirus serum
4768	Rabbit hyperimmune anti - S1 strain BCV serum	Rabbit anti - S1 serum
5000	Gnotobiotic lamb hyperimmune anti - CK and S1 strains BCV serum	Lamb anti - BCV serum
5317	Rabbit hyperimmune anti - S2 strain BCV serum	Rabbit anti - S2 serum
5318	Rabbit hyperimmune anti - CK strain BCV serum	Rabbit anti - CK serum
5319	Rabbit hyperimmune anti - M strain BCV serum	Rabbit anti - M serum
5324	Rabbit hyperimmune anti - PQ strain BCV serum	Rabbit anti - PQ serum
5328	Mouse ascitic fluids containing rotavirus MAbs	Rotavirus MAbs
5453	Rabbit hyperimmune anti - PQ strain BCV serum	Rabbit anti - PQ serum

* Raised by members of the enteritis section, MRI.

Table 2.2

Reference antisera

(Raised by the candidate)

Reference antisera *

Serum number	Description	Serum collected days after first inoculation
5923	Gnotobiotic lamb hyperimmune anti - MI HRT - 18 cell serum	36
5926		43
5919	Gnotobiotic calf hyperimmune anti - S2 strain BCV serum	25
5920		28
5890		50

* Raised by the candidate.

Production of gnotobiotic calf hyperimmune anti-S2 serum

Faeces containing S2 strain BCV were prepared for inoculation by making a 10% suspension in RPMI MM. They were homogenised in Griffiths tubes, and centrifuged at 300g for 15 min at 4°C. The supernate was collected and filtered through a pre-filter, followed by a series of filters with progressively decreasing pore sizes (5 micrometre (um), 1.2um, 0.8um and 0.45um) to ensure bacterial sterility. 19 ml were inoculated orally and a further 5 ml inoculated into each nostril of a 2 day old, male, gnotobiotic calf (day 0).

S2 strain virus grown in cell culture was clarified on a bench centrifuge, concentrated by ultracentrifugation and emulsified with Freund's incomplete adjuvant (FIA) at a ratio (by volume) of virus to adjuvant of 1:2. Emulsification was performed using an homogeniser (Ystral) by dropwise addition of virus to the adjuvant, the mixing vessel being held on ice to prevent overheating. A satisfactory emulsion had formed when it failed to disperse when dropped onto the surface of water. 2.5 and 3.0 ml of this inoculum were given to the calf via the intramuscular (IM) route on days 25 and 35. Serum samples were collected at intervals, and a large volume of serum (serum 5890) was collected on day 50.

Production of gnotobiotic lamb hyperimmune anti-MI HRT-18 cell serum

To prepare the inoculum, MI HRT-18 cells were harvested by freeze/thawing, clarified and concentrated by ultracentrifugation in a manner identical to that described for BCVs. The cell pellets

were emulsified with either Freund's complete adjuvant (FCA) (primary inoculation) or FIA (subsequent inoculations) at a ratio of cells to adjuvant of 1:1 or 1:2. A gnotobiotic lamb was inoculated by the IM route with 1.0 ml, 0.5 ml, 0.75 ml and 2.5 ml of these preparations, on days 0, 15, 26 and 36 respectively. Serum samples were collected on days 0, 7, 14, 26 and 36 (sera 5905, 5906, 5907, 5908 and 5923) and a large volume of serum collected on day 43 (serum 5926). Sera 5923 and 5926 were used as negative control probes in Western blotting experiments.

MONOCLONAL ANTIBODIES

PRODUCTION OF MAbs DIRECTED AGAINST S2 STRAIN BCV

Fusion

Two separate fusions were performed by Miss I. Campbell at MRI to produce hybridoma cells which secreted BCV specific Abs. The fusion protocol used by Miss I. Campbell is briefly summarised below.

Balb/c female mice were inoculated via the intraperitoneal (IP) route with concentrated S2 strain BCV (HA titre = 1280). They were given 100 ul of the virus preparation on 3 separate occasions at intervals of roughly 1 month. The virus was emulsified with FCA for the first inoculation and with FIA for the second and third inoculations. A final injection of 100 ul pelleted S2 strain virus (HA titre = 12800) was given by the IV route 3 days prior to the fusion.

Spleen cells from immunised mice were fused with NS-0 cells in the presence of polyethylene glycol (PEG). The resulting cells

were grown in medium consisting of 2 parts RPMI medium supplemented with 15% heat inactivated FBS and 1% hypoxanthine-aminopterin-thymidine (HAT, Gibco) and 1 part mixed thymocyte medium (MTM, produced by Miss I. Campbell). They were incubated at 37°C in a 5% CO_2/95% air atmosphere. Hybridoma cells were screened for production of BCV specific Abs by testing the supernates in IF, SN and HAI tests. Ab positive cells were frozen in liquid nitrogen prior to being cloned.

Cloning of hybridoma cells

Cloning of hybridoma cells was performed by both Miss I. Campbell and the candidate. Three cell lines were successfully cloned by the candidate, and a further 5 by Miss I. Campbell. Each hybridoma cell line was cloned 3 times by the method of terminal dilution. Supernates were harvested at each stage and checked in the 3 screening tests for the production of BCV specific Abs. Any unstable cells which ceased Ab production were abandoned. During cloning the cells were grown in RPMI medium supplemented with either 10% MTM or heat inactivated FBS at a concentration of 5, 10 or 15%. The precise supplement used depended on the stage of cell growth. The procedures adopted during cell cloning are described below.

The cells were grown in 25 cm^2 plastic tissue culture flasks (Corning). When a sufficient number of cells was present for cloning, the medium was harvested and the cells washed once with PBS. The cells were detached from the flasks by addition of 0.5ml PBS containing 0.0005M versene, and the cells centrifuged at 850g

for 5 min at 4°C. The cells were resuspended in 5.0 ml RPMI medium and an aliquot diluted 1:10 in 0.4% trypan blue stain. Live cells (unstained) were counted and the concentration of the original cell suspension adjusted to 1000 cells/ml. Seven serial doubling dilutions were made from this stock suspension. MTM was diluted 1:10 in RPMI medium and 100 ul added to all the inner wells of a 96 microwell plate (Flow Laboratories). 100 ul of the cell suspensions were added/well. The stock cell suspension (1000 cells/ml) was added to the 10 wells in row B whilst subsequent rows received increasingly dilute cell suspensions. The plate lid was replaced and the cells incubated at 37°C in a 5% CO_2/95% air atmosphere.

Plate wells were examined daily for the presence of cloned hybridoma cells. When a single cell had multiplied to produce a large number of cells, the supernate was harvested and screened for BCV specific Abs. Ab secreting cells were cultured in 24 well tissue culture plates (Costar) and then in 25 cm^2 tissue culture flasks. Some of these cells were frozen in liquid nitrogen and the rest used for subsequent cloning.

Ascitic fluid production

Hybridoma cells which had been cloned 3 times were inoculated into mice for the production of ascitic fluids. The mice were Balb/c or Balb/c cross Swiss male or female mice. A group of about 10 mice were inoculated with each batch of cells. They were primed at least 1 month prior to receiving the cells by the IP inoculation of 0.5 ml 2,6,10,14-tetramethyl-pentadecane (Pristane, Sigma).

Hybridoma cells for inoculation of mice were grown in 75 cm^2 plastic tissue culture flasks (Flow Laboratories). They were harvested as described above and pelleted by centrifugation at 850g for 5 min. The cell pellets were washed 3 times with PBS to remove all traces of FBS and resuspended in 10 ml RPMI (no FBS). The viable cells were counted and the concentrations of the cell suspensions adjusted to 2×10^6 cells/ml. 0.5 ml of these cell suspensions was inoculated via the IP route into each mouse.

The mice were examined daily for the production of ascitic fluids. Mice showing signs of distress were culled and the ascitic fluids collected by opening the abdomens. Mice which developed swollen abdomens in the absence of other clinical signs were tapped using a 23 gauge needle and syringe. The ascitic fluids from each mouse were collected into a 7.0 ml glass bijoux containing 1 drop (50 units) of heparin sodium (Evans).

Harvested ascitic fluids were centrifuged at 850g for 5 min at 4^oC to pellet RBCs and other debris. The clarified ascitic fluids were collected from beneath the surface layer of pristane and screened for BCV specific Abs. Ab positive ascitic fluids which had been produced from the same batch of hybridoma cells were pooled, aliquoted and stored at -70^oC.

Purification of MAbs from ascitic fluids

1.0 ml volumes of ascitic fluids were diluted and filtered through 0.45 um pore size filters. A buffer containing 2M glycine, and 4M NaCl (pH 8.9) was used as the diluent and wash fluid, the volumes used being such that the original ascitic fluids were diluted 1:4 after filtration.

The MAbs were purified by affinity chromatography on a protein A sepharose 4B column (Pharmacia). This procedure was performed by Dr. R. Davies at MRI, and is briefly summarised here. Loading buffer (1.5M glycine, 3M NaCl, pH 8.9) was flushed through the column and the diluted ascitic fluids applied to the top. The unbound fractions (mainly albumin) were washed through with more loading buffer. The bound fractions were eluted off the column with 0.1M citrate solutions of decreasing pHs. The initial solution was at pH 6, subsequent solutions were at pHs 5, 4 and 3. The MAbs eluted off the column at the pHs appropriate to their isotypes (IgG1, IgG2a, IgG2b and IgG3 respectively). Elution of the fractions was monitored by measuring the absorbance at 280 nm. The active fractions were collected in 1M tris (pH 9.0), and sodium azide solution added to give a final concentration of 0.04%.

The purified MAbs were placed in Sartorius collodion dialysis bags and supported in baths of distilled water (DW) by Sartorius filter holders. The MAbs were concentrated by dialysis under vacuum at 4°C. The concentrated MAb solutions were then placed in dialysis tubing (size 1 - 8/32", Medicell International Ltd) and the salt concentrations returned to normal physiological values by dialysing once against DW and then twice against PBS. Each dialysis step was performed over a period of about 8 h at 4°C, and the DW or PBS were stirred throughout.

The purified MAbs were tested for activity in IF, SN and HAI tests and their protein concentrations estimated using Pierce BCA protein assay kits. They were concentrated further if necessary to adjust their protein levels up to at least 1mg/ml.

CVL MAbs

The Central Veterinary Laboratory (CVL), Weybridge, kindly supplied 4 MAbs which had been raised against their own isolate of BCV. This isolate was referred to by us as the CVL strain and the mouse ascitic fluids were identified by the numbers 5528, 5529, 5530 and 5531.

DETERMINATION OF THE MAb ISOTYPES

Binding Site kit

This kit was produced by Binding Site, Birmingham, and was based on the Ouchterlony immunodiffusion technique. The kit contained gels with pre-cut wells set in plastic plates and 6 antisera (typing antisera) directed against the mouse heavy chains IgG1, IgG2a, IgG2b, IgG3, IgA and IgM. 75 ul of test supernates harvested from hybridoma cells grown *in vitro* were pipetted into the central wells of the gels. 10 ul of each of the typing antisera were pipetted into each of the 6 surrounding wells. A positive control serum was included which reacted to give an immunoprecipitation line with each of the typing antisera. RPMI medium supplemented with 5% FBS was used as a negative control. The plates were covered and stored flat at room temperature for 24 h. The gels were then examined with the naked eye for precipitation lines and the results recorded. To increase the sensitivity of the tests, the gels were also stained with Coomassie Brilliant Blue. Prior to staining, the gels were stuck to a piece of gel bond (FMC, Bio-Products) and washed 4 times for 30 min each in PBS. They were dried overnight at 37^{o}C, stained with Coomassie Brilliant Blue for

15 min and destained with ethanol/acetic acid/ DW destainer solution for about 15 min. They were dried at 37°C for about 20 min and examined again for the presence of precipitation lines.

Amersham kit

The Amersham kit was based on the ELISA principle and was performed on typing strips. The supplied strips were impregnated with goat Abs specific for the different classes and subclasses of mouse heavy chains (typing antisera) and had been treated to prevent the non-specific binding of other proteins. The typing strips were incubated at room temperature with 3 consecutive solutions for 15 min each, the strips being washed thoroughly between each stage. The first solutions were the hybridoma cell supernates, tested at dilutions of 1:10. Bound MAbs were detected with horseradish peroxidase (HRP) conjugated sheep anti-mouse Igs. The peroxidase enzymes reacted with the third solution, 4-chloro-1-naphthol, to give purple bands in the regions on the typing strips which corresponded to the isotypes of the MAbs. A positive control mouse Ab was included on the typing strips to check the detection system functioned correctly.

BIOTINYLATION OF MAbs

Purified MAbs were dialysed overnight at 4°C against 0.1M sodium bicarbonate (pH 8.2 - 8.6) and their protein concentrations adjusted to 1 mg/ml. Immediately prior to use, biotin-N-hydroxysuccinimide ester (Sigma) was dissolved in dimethylsulphoxide (DMSO, BDH) to give a concentration of 1 mg/ml.

120 ul of biotin/DMSO were added dropwise to each 1.0 ml of MAb solution, and the reaction mixtures left at room temperature for 4 h. They were dialysed overnight at 4°C against PBS supplemented with 0.02% sodium azide. The biotinylated MAbs were then aliquoted and stored at -20°C prior to use.

TESTS

IMMUNOFLUORESCENT STAINING OF VIRUS INFECTED CELLS

IF staining was used to detect viral antigens in infected cell monolayers as part of several different tests. The exact details of staining varied between the different tests, but the same basic procedure was used throughout.

PBS was used as the diluent and wash fluid during IF staining. The fixed cells were first incubated at 37°C for 30 min with an anti-BCV detecting Ab. This was washed off with PBS and replaced with an anti-species fluorescein isothiocyanate (FITC) conjugate. After incubating at 37°C for a further 30 min the conjugate was washed off and the cells examined for IF under a Leitz UV microscope. When staining cells in 96 well plates, 50 ul of each of the Abs were added to each well and the plates were washed by flooding the wells twice with PBS. When staining cells on coverslips or slides, the cells were stained by flooding with Abs and washed by placing the coverslips or slides in a rack in a bath of PBS. Stained coverslip cultures were mounted in a drop of glycerol (pH 8.4) before examining by UV microscopy.

The Abs used for IF staining varied between the different tests. When mouse MAbs were used as the detecting Abs, bound MAbs were

stained with FITC-conjugated sheep anti-mouse IgG (anti-mouse IgG FITC conjugate, Scottish Antibody Production Unit, SAPU). When rabbit sera were used as the detecting Abs, bound Abs were stained with FITC-conjugated sheep anti-rabbit Igs (anti-rabbit Igs FITC conjugate, Nordic). When bovine or ovine sera were used as detecting Abs, bound Abs were stained with FITC-conjugated pig anti-sheep Igs (anti-sheep Igs FITC conjugate). This latter conjugate was produced by Dr. A. Dawson, MRI and was suitable for staining bovine Abs because it cross reacted strongly with bovine Igs. The optimal Ab dilutions for IF staining were determined by titration. The optimal dilutions of the conjugates varied between batches, but anti-mouse IgG and anti-rabbit Igs FITC conjugates were generally used at dilutions of 1:50, whilst the anti-sheep Igs FITC conjugates were used at dilutions of 1:100.

Monitoring virus growth in coverslip cultures of HRT-18 cells

Viral antigens were detected with lamb anti-BCV serum (serum 5000) used at a dilution of 1:50, followed by anti-sheep Igs FITC conjugate. Virus growth was assessed by comparing the levels of IF present in virus infected and MI HRT-18 cells.

Detection of BCV in nasal epithelial cells

Nasal swab samples were mixed with 1.5 ml VTM on a vortex mixer immediately after collection. 100 ul of the cell suspensions were spun onto clean glass slides in a cytospin apparatus (Shandon Southern) and the cells fixed in acetone. BCV antigens were detected by staining the fixed nasal epithelial cells with lamb

anti-BCV serum (serum 5000) at a dilution of 1:50, rabbit anti-S2 serum (serum 5317) at a dilution of 1:40 or ascitic fluid containing S2/1 MAbs at a dilution of 1:200. Bound Abs were detected with an appropriate anti-species FITC conjugate.

Two controls were included for each sample when serum 5317 was used as the probe Ab. Rabbit anti-rotavirus serum (serum 4381) was used at a dilution of 1:40 as a negative control probe and binding of serum 5317 was blocked by first adding lamb anti-BCV serum (serum 5000) at a dilution of 1:30.

Reading infectivity titrations and SN tests

The detecting serum was lamb anti-BCV serum (serum 5000), used at a dilution of 1:50. Bound Abs were stained with anti-sheep Igs FITC conjugate.

Screening hybridoma cell supernates for BCV specific Abs

50 ul volumes of neat hybridoma cell supernates were added to the wells of standard antigen plates, and bound MAbs detected with anti-mouse IgG FITC conjugate. Each test supernate was scored positive or negative on the basis of the presence or absence of IF. A known positive sample was used as a positive control whilst Mabs were omitted from negative control wells.

MAbs: Patterns of IF and membrane fluorescence

The patterns of IF produced by staining BCV infected HRT-18 cells with MAbs were examined using acetone-fixed standard antigen coverslip cultures. The cells were left unfixed in membrane fluorescence tests.

The coverslips were flooded with MAbs diluted to IF titres of 128. Bound MAbs were detected with anti-mouse IgG FITC conjugate and the cells examined for IF. MI HRT-18 cells were also stained with the MAbs to act as negative controls.

VIRUS TITRATIONS

Infectivity titres (ITs)

Starting from dilutions of 1:10, 10 fold dilutions of the viruses were made in RPMI MM containing 10ug/ml trypsin and incubated at 37°C for 1 h. RPMI GM was removed from confluent monolayers of HRT-18 cells in 96 well plates and replaced by 150 ul/well RPMI MM. 50 ul volumes of the virus preparations were added to duplicate wells. The plates were covered and sealed, and incubated for 2 days at 37°C. The titrations were read by fixing the cells with acetone and staining by IF. Wells containing cells which stained by IF were scored as positive, and the virus IT, log $TCID_{50}$/ml, calculated from the Karber formula (Karber, 1931).

Haemagglutination (HA) titres

HA tests were performed in 96 well plates with "V" shaped wells (Flow Laboratories). Two-fold dilutions of the test virus samples were made in duplicate wells in 50 ul volumes of PBS/0.5% BSA. 50 ul of rat RBCs at a PCV of 0.5% were added to each well, and the plates incubated at room temperature for 1 h. The end points were defined as the reciprocals of the highest dilutions of virus that still gave clear haemagglutination of the RBCs. RBC control wells

which lacked the virus were included to check for non-specific agglutination.

Arklone extraction of organic materials prior to measurement of HA titres

Attempts were made to detect BCV in guts harvested from mice experimentally infected with the virus by measurement of the HA titres of gut preparations. The guts were harvested and homogenised in Griffiths tubes with appropriate volumes of RPMI MM to dilute them 1:10. They were then homogenised with equal volumes of the organic solvent Arklone, to remove non-specific agglutinins. These mixtures were centrifuged at 200g for 5 min at 4°C. This caused the arklone layers to settle at the base of the tubes, covered by a surface layer of debris. The upper aqueous layers were collected and used in HA tests.

ANTIBODY TITRATIONS

IF tests

Doubling dilutions of test polyclonal sera or MAbs were made in PBS in 96 well plates, and 50 ul volumes transferred to duplicate wells of standard antigen plates. The plates were incubated at 37°C for 30 min, unbound Abs washed off with PBS and bound Abs stained by incubating at 37°C for a further 30 min with 50 ul/well of an appropriate anti-species FITC conjugate. The plates were washed again with PBS and examined under a UV microscope. Ab titres were defined as the reciprocals of the highest dilutions which gave clear IF with virus infected cells. A positive control

of known IF titre was included in each test, and each sample was tested with MI HRT- 18 cell control wells to check for non-specific IF.

SN tests

The medium used throughout these tests was RPMI MM. Serum or ascitic fluid samples were first diluted (to 1:10 or 1:40), and heat inactivated at 56°C for 30 min. Hybridoma cell harvests were used neat without heat inactivation and were not titrated out: they were simply scored as being SN positive or negative.

Doubling dilutions of 25 ul volumes of the test samples were made in duplicate in transfer plates (Dynatech). The viruses were diluted to pre-determined concentrations in RPMI MM containing 20 ug/ml trypsin, 25 ul volumes added to each well of the transfer plates and the plates incubated at 37°C for 1 h. The RPMI GM was removed from confluent monolayers of HRT-18 cells in 96 well plates and replaced with 150 ul/well of RPMI MM. The Ab/virus suspensions were added, the plates sealed and incubated at 37°C for 2 days. The cells were then acetone fixed and stained by IF.

Several controls were included in the SN tests. Cell control wells contained RPMI MM only to check that cell morphology was normal and that no contamination had occurred. Virus control wells contained 25 ul of the virus preparations and 25 ul RPMI MM. The average numbers of fluorescent cells per field of view were determined from these wells, and used to define the end points of the tests. The viruses were also titrated to check the susceptibility of the cells to virus infection. The SN titre of a

serum control (serum 5000) was determined in each test, to ensure consistency between the results.

When reading the test, the mean number of fluorescing cells per field of view in the virus control wells was first determined. A field of view was defined as the area outlined by the eyepiece graticule of the microscope. Virus infected cells were often scattered irregularly in the cell monolayer so the fluorescing cells were counted in 5 fields of view and the average taken. The 5 fields of view were at the centre, north, south, east and west positions within the wells. The Ab titres were defined as the reciprocals of the highest dilutions of sera that gave a 90% reduction in the average number of fluorescent cells per field of view. The viruses were used at dilutions which gave between 50 and 200 fluorescent cells per average field of view. The results of each SN test were accepted if the serum control titre was within a 2 fold range of the standard titre.

HAI tests

Polyclonal sera were prepared for HAI tests in 3 stages. Non-specific inhibitors of agglutination were removed by mixing 1.0 ml of the serum samples with 13.0 ml of a 25% suspension of acid washed kaolin in PBS. The preparations were left at room temperature for 30 min and then centrifuged at 200g for 5 min. The supernates were collected and mixed with 1.0 ml of rat RBCs at 40% PCV in PBS/0.5% BSA. They were kept at $4^{o}C$ for 1 h, whilst shaking every 10 min, and centrifuged at 850g for 5 min. Finally the supernates were heat inactivated at $56^{o}C$ for 30 min. Hybridoma cell

supernates and ascitic fluids were not treated prior to being used in HAI tests.

The diluent used throughout the HAI tests was PBS/0.5% BSA. Doubling dilutions of 50 ul volumes of the test samples were made in duplicate in "V" well plates (Flow Laboratories). Viruses of known HA titres were diluted to give 8 HA units and 50 ul added/well. The plates were incubated at 37°C for 1 h, and 50 ul of rat RBCs at 0.5% PCV added to each well. The plates were held at room temperature for a further hour, and the HAI titres determined.

Several controls were included. RBC control wells contained RBCs and diluent only to check for non-specific agglutination. Virus control wells contained doubling dilutions of virus, and diluent instead of the test sera. These wells were used to check that the correct amounts of virus had been used in the tests. Serum control wells contained serum, and diluent instead of the viruses. They were used to check for the presence of non-specific agglutinins in the serum. A standard serum sample was also titrated to check for consistency between tests.

The HAI titres of the test samples were defined as the reciprocals of the highest dilutions of sera which showed clear inhibition of haemagglutination. The results of each test were accepted if the serum control titre lay within a 4 fold range of the standard titre.

ELISAs

Flat bottomed micro ELISA plates (Nunc Maxisorp) were used in

all the ELISAs. The coating Ab buffer consisted of 0.015M sodium carbonate and 0.035M sodium bicarbonate (pH 9.6) (carb/bicarb buffer). Unbound protein binding sites on the plates were then blocked with 2%FBS/PBS/0.05%Tween 20 (T, Sigma) in the post coat stage. The Abs used in the subsequent stages were diluted in PBS/0.05%T, and PBS/0.05%T was also used to wash the plates 3 times after each stage. After the post coat stage the plates were washed only once. The outer wells of the ELISA plates were not used in the tests, but were simply filled with PBS/0.05%T at all stages. At the end of the tests, substrate solution and 2M sulphuric acid were also added to the first column of wells, which served as blanks for zeroing the ELISA reader.

In ELISAs which used a standard antigen preparation, (MAb binding affinity curves, competition ELISAs and epitope blocking asays, EBAs), S2 virus was harvested from infected HRT-18 cells by freeze/thawing and used directly in the ELISAs at an appropriate dilution. MI HRT-18 cell antigens were prepared in a similar manner.

Diagnostic ELISA for detecting BCV

The diagnostic ELISA had been developed by other members of the enteritis section at MRI. It was used in these studies for detecting BCV in faecal samples and for confirming the presence of BCV in TOC harvests with positive HA titres. Faecal samples were prepared in advance by making 1:10 dilutions in PBS/0.05%T and mixing on a vortex mixer. Particulate matter was allowed to settle and the supernates harvested and used in ELISAs. TOC harvests were tested neat or at dilutions of 1:2 in PBS/0.05%T.

The ELISA plates were coated overnight at 4°C with 100 ul/well of coating Ab in carb/bicarb buffer. At the beginning of these studies, sheep anti-BCV (strains S1 and CK) serum (serum 5000) was used as the coating Ab at a dilution of 1:12,800. This was later replaced by MAb S2/1 ascitic fluid, at a dilution of 1:10,000. All subsequent stages were identical between the 2 tests.

After the Ab coating stage, the plates were washed 3 times and post coated with 100 ul/well of 2%FBS/PBS/0.05%T. After standing at room temperature for 1 1/2 h, they were washed once and 100 ul of the test samples added to appropriate wells. The format generally used was to add each test sample to a vertical column of 6 wells. Positive and negative control samples were also included in the tests. The plates were incubated for 3 h at 37°C, washed 3 times and blocking sera added. Wells in rows B and C received 100 ul/well of 2%FBS/PBS/0.05%T, wells in rows D and E received 100 ul/well of a positive block serum and wells in rows F and G received 100 ul/well of a negative block serum. The positive block serum was lamb anti-BCV serum (serum 5000) and the negative block serum was lamb anti-rotavirus serum (serum 3626). Both sera were used at dilutions of 1:20. The plates were incubated at 37°C for 1 h, washed 3 times and 100 ul/well of rabbit anti-PQ serum (serum 5324) added at a dilution of 1:50,000. After incubation at 37°C for a further hour the plates were washed 3 times and 100 ul/well of HRP-conjugated goat anti-rabbit IgG (anti-rabbit IgG HRP conjugate, Wellcome), added at a dilution of 1:6000. The plates were incubated at 37°C for 1 h and washed 3 times.

The substrate working buffer solution was prepared by mixing 12.1 ml 0.091M citric acid solution, 12.9 ml 0.2M disodium hydrogen phosphate solution and 25.0 ml DW. 0.04g of 0-phenylenediamine dihydrochloride (opd, Sigma) and 40 ul of 30% hydrogen peroxide solution were added immediately before use. 100 ul/well of the substrate solution were added to the plates and the colour allowed to develop for about 5 min until the positive control wells were judged by eye to have optical densities (ODs) of about 1.0. The reactions were stopped by the addition of 50 ul/well of 2M sulphuric acid. The OD values were read in a Titertek Multiscan ELISA plate reader using a D492 nm filter. A positive result was recorded if the control wells (rows B and C) and negative block wells (rows F and G) gave OD values greater than 0.1 and the OD of the positive block wells (rows D and E) was reduced by at least 50%.

MAb ELISAs for detecting BCV strain variations

BCV isolates were examined for strain variations in ELISAs using the 8 S2 MAbs in ascitic fluids as coating Abs. The test protocols were similar to that used for the diagnostic ELISA, but the blocking stage was omitted from these tests.

The plates were coated overnight with optimal dilutions of the MAbs in carb/bicarb buffer. The MAbs were washed off and the plates post coated with 2%FBS/PBS/0.05%T. The test samples were diluted in PBS/0.05%T to give HA titres of 32, and 100 ul of each sample added to duplicate wells. Positive and negative controls were included on each plate. The positive control was S2 strain

virus, which had either been grown in TOC or HRT-18 cells, depending on the origin of the test samples. Harvests from MI TOCs or MI HRT-18 cells were used as negative controls. Bound viruses were detected with rabbit anti-PQ serum (serum 5324) followed by anti-rabbit IgG HRP conjugate.

The substrate reactions were stopped when the ODs of the positive control wells (S2 virus) were judged by eye to have reached about 1.0. The mean OD values from the test and control wells were calculated. The test samples were then compared to S2 virus by expressing the OD values obtained with the test samples as %s of the OD value obtained with S2 virus (Dea and Tijssen, 1989a). Non-specific binding of the detecting Abs to MI TOC or MI HRT-18 cell harvests was allowed for by subtracting the mean OD values obtained from the negative control wells from the mean OD values obtained with the test samples and S2 virus. The final equation was therefore:

$$\% \text{ OD test sample} = \left(\frac{(\text{test sample}_{\text{mean OD}} - \text{MI}_{\text{mean OD}})}{(\text{S2}_{\text{mean OD}} - \text{MI}_{\text{mean OD}})}\right) \times 100$$

MAb binding affinity curves

The 8 S2 strain MAbs were titrated in parallel in an ELISA to construct binding affinity curves. The plates were coated overnight at 4°C with lamb anti-BCV serum (serum 5000) diluted 1:6400 in carb/bicarb buffer. They were post coated with 2% FBS/PBS/0.05%T, washed once and S2 virus diluted 1:5 in PBS/0.05%T added to alternate horizontal rows of the plates. MI HRT-18 cell

harvests diluted 1:5 in PBS/0.05%T were added to the remaining wells. The plates were incubated at 37°C for 1 h and washed 3 times. MAbs which had been purified by affinity chromatography from their ascitic fluids were diluted to give protein concentrations of 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.01, 0.005 and 0.001 ug/100 ul. 100ul volumes were transferred to the ELISA plates: each sample was pipetted into 1 well which had received S2 virus and 1 well which had received MI HRT-18 cells. The plates were incubated at 37°C for 1h, washed 3 times and bound MAbs detected with HRP-conjugated sheep anti-mouse gamma-globulin (anti-mouse gamma-globulin HRP conjugate, SAPU), used at a dilution of 1:30. The plates were incubated for a further hour at 37°C, washed 3 times and opd substrate solution added. The reactions were stopped after 4 min and the OD values read. Binding affinity curves were constructed by plotting the OD values against $\log_{10}$(ug MAb/well).

Competition ELISAs

MAbs which had been purified by affinity chromatography from their ascitic fluids were used at known Ig concentrations as competitor Abs in competition ELISAs. The purified MAbs were biotinylated for use as detecting Abs in competition ELISAs and EBAs.

Plates were coated overnight at 4°C with lamb anti-BCV serum (serum 5000) at a dilution of 1:6400 and post coated with 2% FBS/PBS/0.05%T. S2 virus or MI HRT-18 cell harvests were added to appropriate wells. They were either used neat or at dilutions of

1:4, depending on whether biotinylated N or HE/S MAbs respectively were being used as detecting Abs. The plates were incubated for 1 h, washed and standard dilutions of competitor MAbs added. Negative control wells received 2%FBS/PBS/0.05%T in place of the competitor Abs. After incubating for a further hour the plates were washed and pre-determined optimal dilutions of biotinylated detecting MAbs added. The plates were incubated for an hour, washed and incubated for a further hour with appropriate dilutions of avidin/HRP conjugate. The opd substrate solution was added and the reactions stopped when the colour intensity in the negative control wells (no competitor) was judged by eye to have reached an OD of about 1.0.

The ODs were read on the ELISA plate reader and the average OD of the negative control wells calculated ($OD_{\text{no competitor}}$). The N MAbs gave higher levels of non-specific binding to MI HRT-18 cells than the S and HE MAbs, and this was allowed for in these tests by subtracting the mean ODs obtained in the MI HRT-18 cell wells from all OD values used in subsequent calculations. At each value of ug competitor MAb/well the percentage competition was calculated from:

$$\% \text{ competition} = \frac{(OD_{\text{no competitor}} - OD_{\text{with competitor}})}{OD_{\text{no competitor}}} \times 100$$

Each test was performed on 2 separate occasions and the average % competition values calculated. A series of competition curves were constructed for each biotinylated detecting MAb by plotting the % competition values against $\log_{10}$ (ug competitor MAb/well).

Epitope blocking assays

EBAs were performed to quantify the Abs present in polyclonal sera which were directed against individual viral proteins and epitopes. The test formats of these assays were identical to those previously described for competition ELISAs, except that polyclonal sera were used in place of competitor MAbs.

A mean $OD_{(no\ competitor)}$ value was obtained for each test plate from a set of 11 wells to which PBS/0.05%T was added in place of the competitor Abs. From this value a competition end point OD value was determined. For the S and HE MAbs, the end point OD was defined as 50% the mean $OD_{(no\ competitor)}$ value. The N MAbs gave lower levels of self-competition in competition ELISAs, so the end point ODs in these EBAs were defined as 65% and 80% for MAbs S2/6 and S2/8 respectively. Test samples were titrated in duplicate at 5 standard dilutions and OD values obtained. The mean OD values were plotted against the serum dilutions and the dilutions at the end point ODs determined. The reciprocals of these values were a measure of the Abs present in the test samples.

ANALYSIS OF PROTEINS

PROTEIN ESTIMATIONS

Protein estimations were performed using Pierce BCA Protein Assay kits. The tests were performed in flat bottomed 96 well microtitre plates (Nunc). These assays were based on the ability of proteins to reduce Cu^{2+} ions to Cu^{1+} ions, which then reacted with bicinchoninic acid to give purple solutions whose ODs were measured on a Multiscan ELISA plate reader using a 562 nm filter.

Standard curves were constructed from the readings obtained with bovine albumin solutions of known concentrations, and the protein concentrations of the test samples calculated from these curves.

WESTERN BLOTTING

The proteins were separated by Laemmli SDS-PAGE, electrophoretically transferred to nitrocellulose membranes and then probed with either polyclonal sera or MAbs (Laemmli, 1970; Towbin et al, 1979; Burnette, 1981; Gershoni and Palade, 1983).

Gels

Gels were run in a Biorad Protean II apparatus, assembled using 0.75 mm spacers. The gels consisted of 10% polyacrylamide resolving gels and 3% polyacrylamide stacking gels, with 14.0 cm length troughs for the test samples and 0.4 cm edge wells for the MW markers. The test samples were prepared by boiling 200 ul of the sample with 200ul 2 x Laemmli sample buffer (with or without ME) (see Appendix) for 90 sec. 10 ul aliquots of the standard MW markers (Sigma, MW-SDS-70 kit) were also boiled for 90 sec. The samples were loaded onto the gels supported in tanks filled with Laemmli electrode buffer (see Appendix) and connected to Bio-Rad Model 200/2.0 power packs. The gels were run at 200 volts (v) with a current limit of 0.04 amperes (amps) for about 4 h until the dye fronts had moved about 14 cm through the resolving gels.

Transfer of proteins to nitrocellulose paper

The proteins separated by PAGE were transferred to 0.2 um pore

size nitrocellulose membranes (Sigma) in a trans-blot electrophoretic transfer cell (Bio-Rad) containing tris/glycine electrode blotting buffer (see Appendix). The apparatus was connected to a Bio-Rad Model 200/2.0 power pack, and transfer carried out overnight at 60v with a current limit of 1.00 amp. A magnetic bar stirrer and cooling coils containing circulating cold water were included in the transfer cell.

After transfer, the gels were stained for about 2 1/2 h in Coomassie Brilliant Blue stain and then destained for about 2 h in several changes of acetic acid/alcohol/DW destainer solution (see Appendix). The stained gels were examined to check that most of the proteins had been successfully transferred. The nitrocellulose sheets were stained with 0.1% Ponceau S (Sigma) in DW for about 4 min and destained for about 3 min in several changes of DW. The Ponceau S stained all the proteins a deep pink colour. The stained nitrocellulose sheets were examined to check that the proteins had transferred evenly, and any areas where transfer was poor were marked and not used in subsequent probing experiments. The strips of nitrocellulose bearing the standard MW markers were removed and later used to calculate the MWs of unknown proteins. The rest of the nitrocellulose sheets were either probed immediately or kept moist with PBS in sealed plastic bags and stored at $4^{o}C$.

Probing the nitrocellulose-bound proteins

PBS supplemented with 0.5%T (PBS/0.5%T) was used as the blocking and washing solutions and as the diluent for the Abs. All incubation stages were performed at room temperature on a rotary shaker.

The unbound protein binding sites on the nitrocellulose paper were first blocked by incubation in PBS/0.5%T for 1 h. Either one of two methods was then used to probe the blotted proteins. In the first method the nitrocelluose sheets were cut into strips. Each strip was placed in a tube containing diluted probe Ab and incubated for 1 h. The strips were rinsed 3 times and washed 3 times (5 min per wash) in PBS/0.5%T. Bound mouse MAbs and rabbit polyclonal Abs were detected by addition of 168 kilobequerel (KBq) of ^{125}I Protein A in PBS/0.5%T. After incubation for 1 h the strips were again rinsed 3 times and washed 3 times in PBS/0.5%T, and mounted on filter paper for autoradiography. This was performed using Kodak Xomat 'S' film with a Dupont 'lightening plus' intensifying screen. The casettes were stored at -70^{o}C and the films devloped after a suitable period of time.

In the second method the nitrocellulose sheets were tightly clamped in an Immunetics Miniblotter 25. The probes were added to appropriate channels, care being taken to avoid introducing air bubbles and causing cross contamination between adjacent channels. After incubating for 1 h the probe Abs were flushed out of the apparatus with 1 l of PBS/0.5%T. The nitrocellulose sheets were removed from the miniblotter, rinsed twice and washed 3 times in PBS/0.5%T. Bound MAbs were detected with anti-mouse gamma-globulin HRP conjugate. Bound bovine or ovine Abs were detected with HRP-conjugated donkey anti-sheep/goat IgG (anti-sheep IgG HRP conjugate, SAPU), which cross reacted strongly with bovine Abs. The nitrocellulose sheets were incubated for 1 h in appropriate dilutions (1:100) of the conjugate, rinsed 3 times and washed 3

times, and bound conjugate detected with 4-chloro-1- naphthol substrate solution. This was prepared by dissolving 0.06g 4-chloro-1-naphthol (Sigma) in 20 ml ice cold methanol (solution A). 100 ml of a 0.02M Tris, 0.5 M NaCl solution were prepared, and 60 ul ice cold 30% hydrogen peroxide solution added immediately before use (solution B). Solutions A and B were mixed at room temperature and immediately poured onto the nitrocellulose sheets. The colour was allowed to develop for 10-30 min, and the reaction stopped by washing with DW. Probed blots were mounted on filter paper and photographed the same day before fading could occur.

RADIOLABELLING/RADIOIMMUNOPRECIPITATION

Radiolabelling virus proteins

The media used throughout radiolabelling experiments were supplemented with trypsin at 1 ug/ml and actinomycin D at 5 ug/ml.

S2 strain BCV was treated for 1 h at 37°C with 10 ug/ml trypsin. The RPMI GM was removed from confluent monolayers of HRT-18 cells grown in 24 well tissue culture plates (Costar). The cells were washed twice with PBS and inoculated with virus at an moi of 10. Negative control cells were also infected with a mock inoculum. After incubating at 37°C for 1 h, the inocula were removed and the cells washed twice with PBS. 1.5 ml of RPMI MM were added to each well, and the cells incubated at 37°C for 3 h. The RPMI MM was then replaced with methionine free MEM and the cells incubated for a further hour.

At 5 h pi, 3 MBq of ^{35}S-methionine were added to appropriate wells. Unlabelled methionine was also added in some experiments.

The cells were incubated at 37°C for appropriate lengths of time, and the radiolabelled materials harvested. The supernates were collected directly and stored at -70°C for use in subsequent tests. The cells were first washed twice with PBS and scraped off into RIPA buffer (see Appendix) at 4°C. This was then clarified by centrifugation at 100,000g for 60 min in an SW55 rotor on a Beckman L2 or L5 ultracentrifuge, and the supernates (cell lysates) and cell pellets collected and stored at -70°C. The level of incorporation of radiolabel was monitored by scintillation counting and by autoradiographic detection of proteins separated on gels. The samples were subsequently used in RIP tests.

During the radiolabelling experiments, the ^{35}S-methionine was omitted from a few wells of virus infected wells, which served as controls to check for virus growth. The supernates were harvested and the ITs measured, whilst the cells were fixed with acetone and virus detected by IF staining.

Testing ^{35}S-methionine incorporation: scintillation counting

2.5 ul samples were pipetted onto 2.5 cm diameter Whatman glass microfibre filter discs. They were boiled for 5 min in 5% trichloroacetic acid (TCA) containing 3% casamino acids (cas-AAs, Difco). The filters were then washed once in 5% TCA/3% cas-AAs, twice in ethanol and twice in ether. They were dried, put into vials, and 1.0 ml scintillation fluid (0.018M PPO, 2,5-diphenyloxazole, BDH and 0.0005M POPOP, 1,4-Di-2- (5-phenyloxazolyl)-benzene, BDH) added to each vial. They were

counted for 1 min on a scintillation counter, each sample being counted twice and the mean taken.

Testing ^{35}S-methionine incorporation: protein gels

20 ul samples at 1 x 10^6 counts/min were boiled for 2 min with 5 ul cracking buffer (10% SDS, 25% ME, 50% glycerol). The samples were run on protein gels, the gels dried and processed for autoradiography.

Radioimmunoprecipitation, RIP

Aliquots of test samples (radiolabelled virus or MI HRT-18 cells) containing 4 x 10^6 counts/min were diluted in RIPA buffer (see Appendix) /0.5% SDS to a volume of 75 ul. They were mixed on a vortex mixer with 25 ul rabbit anti-S2 strain BCV serum (serum 5317) and left at 4^{o}C overnight. 50 ul aliquots of 100 mg/ml protein A sepharose (Pharmacia) in RIPA buffer were also left at 4^{o}C overnight.

The virus/Ab mixtures were mixed with the aliquots of swollen protein A sepharose and left on a rocker at 4^{o}C for 2h. The protein A sepharose complexes were pelleted by spinning at 17,000g for 1 min on a Centra-M high-speed microcentrifuge. The pellets were washed 3 times with ice cold RIPA buffer/0.1% SDS and re-pelleted. They were boiled for 2 min with equal volumes of 2 x LSB containing 2% SDS and 4% ME, run on protein gels and precipitated radiolabelled proteins detected by autoradiography.

Protein gels: Radiolabelling/RIP experiments

Radiolabelled proteins were run on protein gels and detected by autoradiography in order to monitor the success of radiolabelling experiments and to detect precipitated proteins after RIP.

3% stacking and 7.5% resolving polyacrylamide gels were set up in a BRL gel apparatus, using 1.5 mm spacers and a 20 tooth comb. The samples were prepared by boiling for 2 min with the appropriate buffer and loaded onto the gels. The apparatus was connected to a power pack (Shandon Vokam 400) and the gels run at 0.04 amp for about 4 h.

Gels were stained overnight in Coomassie Brilliant Blue. They were destained in several changes of destainer solution and soaked in Amplify (Amersham) for 30 min. The gels were washed again for 30 min in destainer solution supplemented with 3% glycerol. They were then mounted on filter paper and dried in a vacuum drier. Autoradiography was performed using Kodak Xomat 'S' film with a Dupont 'lightening plus' intensifying screen. The casettes were stored at -70^{o}C and the films developed after appropriate lengths of time.

CALCULATION OF THE MWs OF PROTEINS SEPARATED BY SDS/PAGE

Standard MW markers (Sigma, MW-SDS-70 kit) were included on all protein gels. They were either stained on the gels with Coomassie Brilliant Blue or transferred to nitrocellulose paper and stained with Ponceau S (0.2% in DW). The relative mobility (Rf) of each of the MW markers was calculated from:

$$Rf = \frac{\text{distance of MW marker migration from top of gel}}{\text{distance of tracking dye migration from top of gel}}$$

The $\log_{10}$ MWs of the markers were plotted against the Rf values, to give straight line calibration curves. The Rf values of the unknown proteins were calculated and their MWs deduced from the standard curves.

ANIMAL EXPERIMENTS

MURINE MODEL

Trials were performed to establish whether neonatal mice were suitable to use as experimental models of BCV infection. The mice used throughout these tests were Swiss White mice, aged between 2 and 8 days. They were kept under conventional conditions with their dams.

The baby mice from all the litters were pooled prior to inoculation. Individuals were then selected at random, inoculated and returned to a mother mouse. Each mother mouse received a similar number of baby mice (generally 8-10). Weak or small baby mice were not used in these experiments. Test mice were each given 0.1 ml of either virus infected or MI HRT-18 cells. The inocula were prepared by freeze/thawing infected cells. These preparations were then either used directly or after clarification and concentration in an ultracentrifuge. The inocula were deposited into the top of the oesophagus, using a syringe attached to a 23 gauge needle with a plastic tip. Control mice were not given any inoculum.

The baby mice were monitored clinically each day for up to 6 days pi. The abdomen of each mouse was gently squeezed to check for the presence of diarrhoea, and any affected mice were marked

with a pen. Litters of mice which had been inoculated with virus were always examined last, to prevent transmission of virus between test groups. In some experiments the baby mice were kept alive throughout the trial. In other experiments, mice which developed diarrhoea were culled, and the guts (duodenum to rectum) harvested. The guts were weighed, and appropriate volumes of RPMI MM added to dilute them 1:10. They were homogenised in Griffiths tubes, and the resulting suspensions centrifuged at 200g for 5 min at 4°C. The supernates were collected and attempts made to detect BCV by measuring their ITs in HRT-18 cells and by measuring their HA titres with rat RBCs.

GNOTOBIOTIC ANIMALS (calf, lamb and piglet)

The lamb was delivered by hysterectomy and the calf and piglet by hysterotomy performed by the Clinical Department at MRI into sterile environments. The animals were maintained in positive pressure isolators supplied with filtered air and cared for by staff belonging to the Gnotobiotic Animal Unit. The animals were fed on tinned, sterilised milk and handled through sleeves and gloves attached to the isolators. All materials entering or leaving the isolation pens did so through an entry port with inner and outer doors. Incoming materials were autoclaved whenever possible, sprayed with 0.3% peracetic acid and allowed to stand for about 30 min in the entry port chamber before entering the isolation pen.

Inoculation of the gnotobiotic calf and lamb has already been described under the heading 'Production of polyclonal sera'. The

gnotobiotic piglet received 10 ml orally and a further 1.0 ml up each nostril of a preparation of faeces containing S1 strain BCV. The inoculum was prepared by making a 1:5 dilution of the faeces in PBS, homogenising in a Griffiths tube and centrifuging at 300g for 15 min at $4^{o}C$.

Measurement of % dry matter (% DM) of calf faecal samples

Representative samples of faeces were placed in tin foil dishes. The dishes were weighed before and after addition of the faeces and the mass/g of the wet faecal samples calculated. The faeces were dried to constant weight in a hot air oven. The mass/g of the dry faecal samples were calculated, and the % DM of the original faecal samples found from:

$$\% \text{ DM} = \frac{\text{mass/g of dry faeces}}{\text{mass/g of wet faeces}} \times 100$$

CHAPTER 3

ISOLATION AND GROWTH OF BOVINE CORONAVIRUSES

CHAPTER 3
ISOLATION AND GROWTH OF BOVINE CORONAVIRUSES

Introduction

BCVs are notoriously difficult to isolate and grow in cell culture (Bridger et al, 1978b; Hajer and Storz, 1978). This has meant that studies to determine the extent of strain variation have been limited to examining a relatively small number of isolates. A more efficient method of in vitro growth of BCV would enable a more comprehensive examination of strain variation to be undertaken.

Tracheal organ cultures (TOCs) derived from bovine foetuses and newborn calves have been used for the primary isolation and growth of BCVs derived from diarrhoeic calf faeces and respiratory tract material (Stott et al, 1976; Bridger et al, 1978b; Thomas et al, 1982; McNulty et al, 1984). Several continuous cell lines including BEK-1, Vero, MDBK and PK-15 cells have been used for the in vitro growth of BCV (Inaba et al, 1976; Dea et al, 1980a). HRT-18 cells have proved to be particularly susceptible to BCV infection (Laporte et al, 1979; Vautherot, 1981; King et al, 1985).

Attempts were made to isolate Scottish field BCV strains from faecal samples in TOCs obtained from bovine foetuses and neonatal calves. Some of these viruses were then adapted to growth in HRT-18 cells. The growth kinetics of S2 strain BCV in HRT-18 cells were studied to define the optimum times for harvesting and radiolabelling the virus.

Results

Faecal samples

Faecal samples from diarrhoeic calves were sent from the

Scottish VI Centres to the MRI for BCV diagnosis by ELISA. The results obtained from 1987 to 1989 are summarised in Table 3.1. BCV positive samples were stored at -70°C.

Isolation of BCVs in tracheal organ cultures

BCVs were isolated from the faecal samples in TOC. The tracheas were obtained from bovine foetuses (mid to late gestation) and conventional calves (approx 1 to 70 days old). Most of the calves were bought from markets, so their histories (whether colostrum deprived or colostrum fed) were unknown. Calves which had diarrhoea and which were found by ELISA to be excreting BCV in their faeces were not used as donors. Each faecal sample was inoculated onto tracheal rings in 2 separate petri dishes. To prevent cross contamination, each pair of petri dishes was incubated in a separate sealed plastic container. Uninfected control cultures were also prepared. The medium bathing the tissues was harvested twice per week, pooled for each pair of petri dishes, and BCV growth monitored by measurement of the HA titres.

The HA titres of the harvests obtained from viruses isolated in TOCs derived from bovine foetuses and neonatal calves are summarised in Tables 3.2 and 3.3. Peak HA titres were generally obtained in the first three harvests and declined to low levels by the fifth harvest. The precautions taken to avoid cross contamination of viruses between cultures were successful as no HA activity was ever observed in any of the uninfected controls. A few cultures which succumbed to bacterial or fungal contamination were immediately discarded.

Table 3.1

Detection of BCV in faecal samples

Detection of BCV in faecal samples

Year	Samples +ve*	Samples tested	% samples +ve
1987	4	38	10.5
1988	37	591	6.2
1989	116	880	13.2

* Number of faecal samples which gave a positive result on a diagnostic ELISA for BCV.

Table 3.2

Isolation of BCV in bovine foetal TOCs

Faecal sample	HA titres* of harvest numbers : 1	2	3	4
L1209	1	8	16	4
L2755	16	8	4	1

*HA titres are expressed as the reciprocals of the highest dilutions of harvests which gave clear haemagglutination with rat RBCs.

Table 3.3

Isolation of BCV in calf TOCs

Faecal sample	HA titres* of harvest numbers : 1	2	3	4
K2595/1	32	>128	>128	16
K2595/2	>128	>128	>128	64
L1096	–	64	>128	>128
L1121	16	>128	64	64
L1209	64	32	32	32
L1217	64	64	32	32
L1280/2	>128	64	64	32
L1354/2	32	>128	64	32
L3032	16	>128	>128	>128
L3080	64	32	64	32
L3140/1	64	>128	c	c
L3140/2	32	64	>128	c
L3140/3	–	>128	>128	64
L3228	4	64	>128	64
L3275	64	64	>128	16
L3318	64	32	c	c
L3352	64	64	64	32
L3372	-	16	>128	c
L3472	64	64	c	c
N164	>128	>128	64	c
N225	32	64	64	32
N339	>128	>128	32	32
N539	2	32	>128	16
N662	1	>128	64	32
N710	32	>128	32	8
N1193	64	>128	64	32
N1258/2	64	>128	64	64
N1334	32	c	c	c
0011	64	64	64	64
Mean titre	57	112	106	51

*HA titres are expressed as the reciprocals of the highest dilutions of harvests which gave clear haemagglutination with rat RBCs.
- indicates HA titres < 1.
c = culture contaminated with bacteria or fungi.

Tracheal organ cultures derived from bovine foetuses were compared with those derived from neonatal calves for their ability to support the isolation of BCVs. Eight faecal samples were simultaneously inoculated into both types of culture (Table 3.4). The calf TOC proved more successful as BCVs from 3 of the inoculated samples grew to HA titres of 64 in calf TOCs whilst BCVs from only 2 samples grew to maximal HA titres of 16 in foetal TOCs.

Table 3.5 summarises the results for virus isolation in TOC. A total of 17 samples was inoculated onto organ cultures derived from 5 foetuses. Only 2 (12%) of these samples grew to HA titres of 16. Sixty samples were inoculated onto organ cultures derived from 6 neonatal calves. Twenty-nine (48%) grew to HA titres of at least 16 and all but one of these samples grew to HA titres of at least 64. These results clearly demonstrate that tracheas taken from neonatal calves are more suitable for isolation of BCVs than those obtained from bovine foetuses.

The ages of the donor calves and their SN titres to BCV are summarised in Table 3.6. All were Jersey bull calves. They varied in age from 1 day to 10 weeks old, but age had no apparent effect on the isolation rates of BCV. Their SN titres to BCV may have been more important as lower rates of virus isolation were achieved using tracheas from calves with high Ab titres. Unfortunately insufficient data were available for statistical analysis to determine whether the % of BCVs isolated in TOC could be predicted from the SN titre of the donor calf.

Table 3.4

BCV isolation in TOC

Comparison of tracheas from neonatal calves and bovine foetuses

Samples inoculated onto both types of TOC

Faecal sample	Source of trachea**	HA titres* of harvest numbers : 1	2	3	4
L1096	F	–	–	–	–
	C	2	64	32	16
L1121	F	1	–	–	–
	C	32	64	32	8
L1209	F	1	8	16	4
	C	8	64	16	c
L1242	F	–	–	–	–
	C	–	–	–	–
L1243	F	–	–	–	–
	C	–	–	–	–
L1275	F	–	–	–	–
	C	–	–	c	c
L2755	F	16	8	4	1
	C	4	c	c	c
L2793	F	2	–	–	–
	C	2	–	–	c

*HA titres are expressed as the reciprocals of the highest dilutions of harvests which gave clear haemagglutination with rat RBCs.

** F = bovine foetus C = calf

- = HA titres <1.

c = culture contaminated with bacteria or fungi.

Table 3.5

BCV isolation in TOC

Comparison of tracheas from neonatal calves and bovine foetuses: Overall results

BCV isolation in TOC

Comparison of tracheas from neonatal calves and bovine foetuses

Overall results

Source of trachea	Number of tracheas used	Number of samples inoculated	Growth of BCV to HA titres* of : HA > 16		HA > 64	
			Samples**	%	Samples**	%
Bovine foetus	5	17	2	12	0	0
Neonatal calf	6	60	29	48	28	47

*HA titres are expressed as the reciprocals of the highest dilutions of harvests which gave clear haemagglutination with rat RBCs.

** Number of samples inoculated onto TOC which grew to give at least 1 harvest with an HA titre of >16 or >64.

Table 3.6

Isolation rates of BCV in calf TOCs

TOC	Calf		Isolation of viruses in TOC*		
	Age/d (approx)	SN titre to BCV	Viruses isolated	Samples inoculated	% viruses isolated
1	1	<100	5	8	63
2	2	NT	9	14	64
3	2	800	3	13	23
4	14	2263	3	8	38
5	35	<100	9	15	60
6	70	<100	4	8	50

* Viruses were considered to have been isolated if the samples inoculated onto TOC grew to give at least 1 harvest with an HA titre of >16.

NT = not tested.

All TOC harvests with HA titres greater than 32 were tested using the diagnostic ELISA. These samples all gave positive results as high OD values (range 0.57 to 1.95) were obtained and binding of the detecting Ab was blocked by lamb anti-BCV serum (serum 5000). Negative controls consisted of harvests from both uninfected cultures and infected cultures with no detectable HA activities. These samples all gave very low OD readings (range 0.07 to 0.10) on the ELISA. These results confirmed that the HA activities of the TOC harvests were due to the isolation of BCV from the inoculated faecal samples.

Harvests obtained from each inoculated faecal sample with HA titres of at least 32 were pooled and stored at -70^{o}C. They were identified by the original faecal sample number followed by "TOC 1" to indicate the virus had been passaged once in TOC. Twelve TOC 1 viruses were passaged onto a second set of TOCs (TOC 2). They all grew to an HA titre of at least 128 (results not shown).

Adaptation of BCVs to growth in HRT-18 cells

Attempts were made to adapt 12 of the viruses isolated in TOC to growth in HRT-18 cells. Initial passages were performed in HRT-18 cells grown on coverslips. Two tubes were inoculated with each virus and MI control tubes also set up. The tubes were harvested after 2 or 3 days, and virus growth monitored by examination of the cells for CPE, IF staining of acetone fixed cells and measurement of HA titres. A clearly recognisable CPE was never observed in any of the cultures but a few syncytia were seen occasionally. The levels of IF were scored by eye as low (+), moderate (++) or high

(+++). Viruses failing to produce a moderate number of IF positive cells after a few passages were abandoned.

Viruses growing in coverslip cultures of HRT-18 cells were then passaged in HRT-18 cells grown in 75 cm^2 flasks. Growth was monitored by measurement of the HA and infectivity titres. Seven of the 12 viruses (58%) were successfully adapted to growth in HRT-18 cells (Table 3.7).

Growth curves: S2 strain BCV in HRT-18 cells

HRT-18 cells were infected with S2 strain BCV at an moi of 0.5 or 2.0. Virus was harvested at various times pi and titrated in HA and infectivity tests. When an moi of 0.5 was used, both total virus (released after freeze/thawing the cells and medium) and free virus (in clarified medium) were assayed (Fig 3.1). When an moi of 2.0 was used, only total virus was assayed (Fig. 3.2).

The growth curves obtained using mois of 0.5 or 2.0 were very similar. Production of infectious virus commenced at 6-9 h pi and peak titres were reached at about 30 h pi. The IT of total virus increased before that of free virus due to the slight delay in virus release from infected cells. HA titres rose (at 48 h when moi = 0.5 and 24 h when moi = 2.0) and peaked (at 77 h when moi = 0.5 and 54 h when moi = 2.0) later than infectivity titres. This test failed to detect any differences in the HA titres of total and free virus.

Discussion

Faecal samples

BCV was detected in 10% of the faecal samples collected from

Adaptation of TOC isolates to growth in HRT-18 cells

Isolate	Test : IF* IT** HA titre	Passage no. in HRT-18 cells 1	2	3	4	5
L1096	IF	+	–	–		
	HA titre	NT	NT	NT		
L1121	IF	+	+	+	++	+
	HA titre	NT	NT	NT	NT	NT
L1209	IF	+	+	+	NT	+
	HA titre	NT	NT	NT	NT	NT
L1217	IF	–	–	+		
	HA titre	8	2	0		
L3080	IF	+	+	++	NT	NT
	IT	NT	NT	NT	3.8	5.3
	HA titre	16	4	4	16	32
L3352	IF	–	++	++	++	NT
	IT	NT	NT	NT	NT	6.8
	HA titre	8	2	8	4	64
L3372	IF	–	NT	+++	NT	NT
	IT	NT	NT	NT	5.8	5.3
	HA titre	2	8	8	32	32
N164	IF	–	+	+	+	+++
	HA titre	16	4	0	2	64
N225	IF	++	++	NT	NT	
	IT	NT	NT	6.8	5.8	
	HA titre	16	16	>128	32	
N539	IF	++	++	+++	NT	NT
	IT	NT	NT	NT	5.8	5.8
	HA titre	16	8	4	32	>128

Table 3.7 (continued)

Isolate	Test : IF* IT** HA titre	Passage no. in HRT-18 cells 1	2	3	4	5
N662	IF	–	–			
	HA titre	8	NT			
0011	IF	–	++	++	NT	NT
	IT	NT	NT	NT	5.3	4.3
	HA titre	8	32	16	32	32

*IF = number of cells stained by indirect immunofluorescence scored as none (-), low (+), moderate (++), or high (+++).
**IT = log TCID50/ml.
NT = not tested.

Figure 3.1

Growth curve: S2 BCV in HRT-18 cells

moi = 0.5

Growth curve : S2 BCV in HRT-18 cells
moi = 0.5

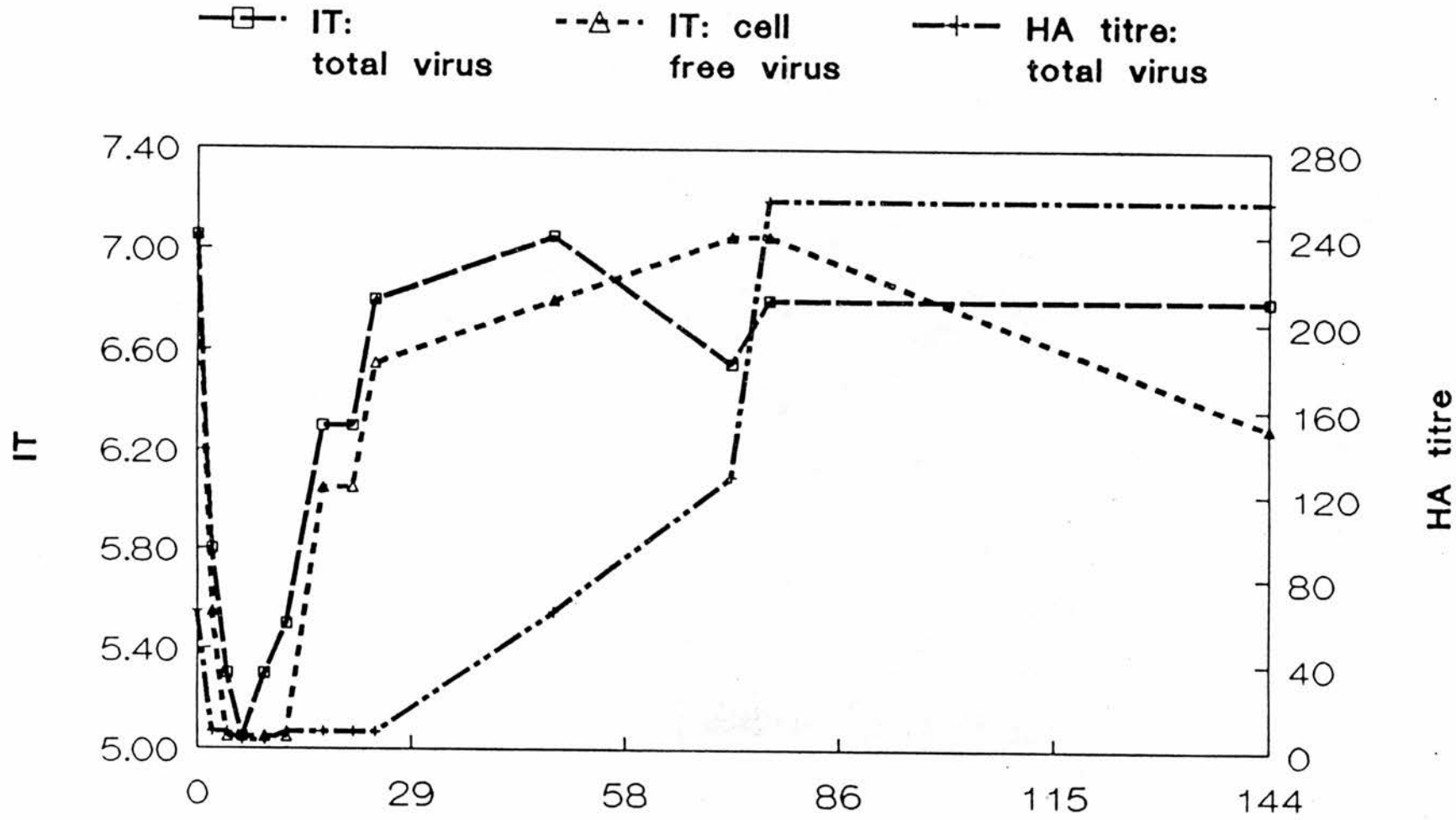
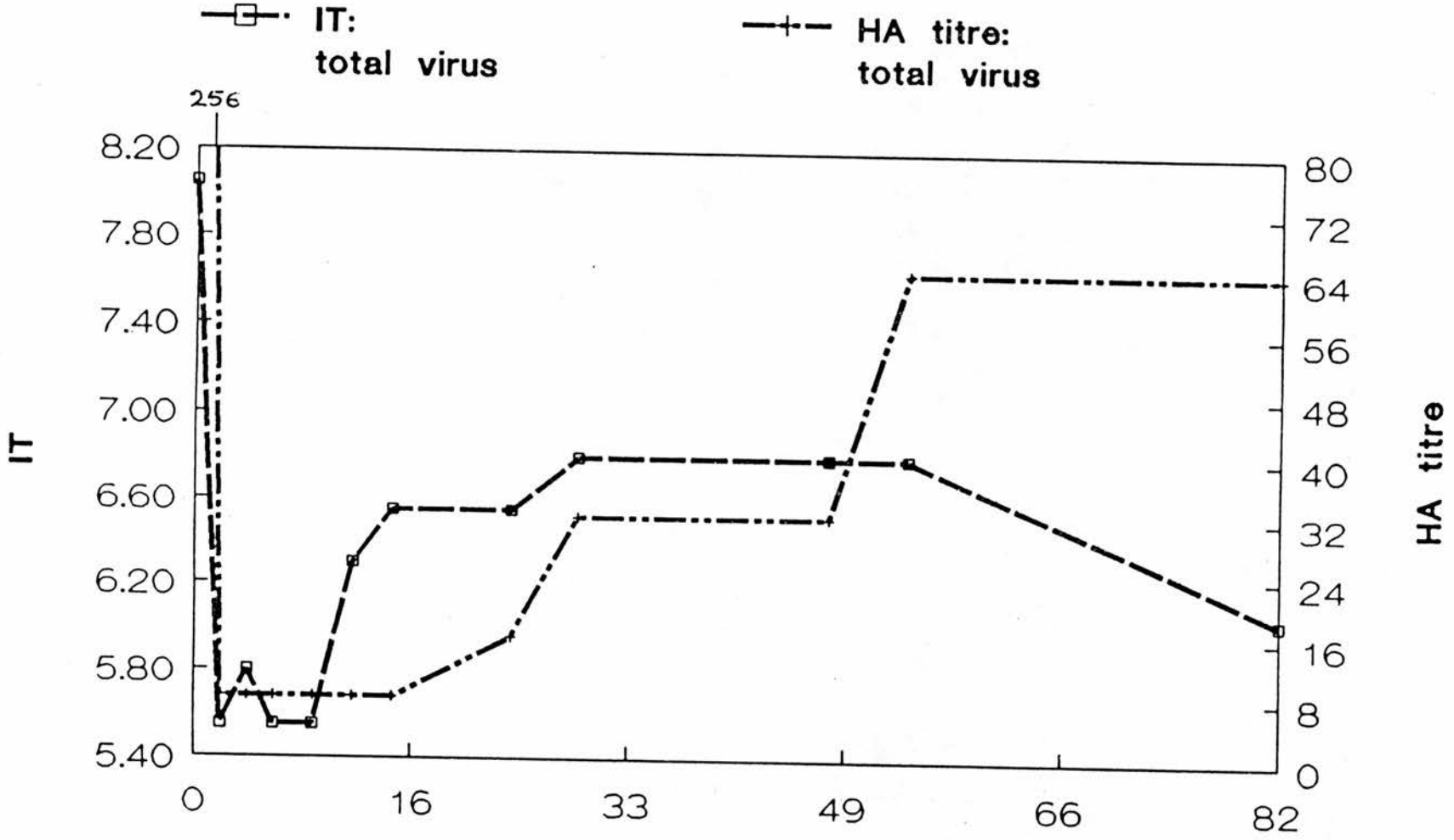


Figure 3.2

Growth curve: S2 BCV in HRT-18 cells

moi = 2.0

Growth curve : S2 BCV in HRT-18 cells
moi = 2.0



diarrhoeic calves between 1987 and 1989. These samples were highly selected due to the system of referral to MRI, but the results are comparable with those obtained in 2 surveys carried out in 1986. Reynolds et al (1986) detected BCV in the faeces of 69 out of 490 (14%) diarrhoeic calves in southern Britain and Wales. Snodgrass et al (1986) detected BCV in the faeces of 24 out of 302 (8%) diarrhoeic calves in northern England and Scotland.

There was a large increase in the number of samples submitted to MRI for BCV diagnosis between 1987 and 1989. This was probably because the success of Rotavec K99 vaccine in preventing diarrhoea caused by E. coli and rotavirus infections led to an increased awareness of the other causes of neonatal calf diarrhoea. Prior to this vaccine becoming available, concurrent rotavirus and BCV infections were common in diarrhoeic calves, and may have resulted in more severe clinical disease (Babiuk et al, 1985; Reynolds et al 1986; Snodgrass et al, 1986). The effects of controlling rotavirus diarrhoea on BCV infections are at present unknown.

Isolation of BCVs in tracheal organ culture

TOCs derived from conventional calves proved to be a highly efficient system for primary isolation of BCVs from diarrhoeic calf faeces. The cultures remained viable for at least 17 days, viruses grew to high HA titres in their first passage (many $\geq$ 128) and viability of these viruses was confirmed by further passage. Measurement of HA titres provided a rapid and simple method for monitoring large numbers of culture harvests. ELISAs confirmed that the HA activities of the harvests were due to BCV.

Attempts to isolate BCV in TOCs derived from bovine foetuses proved less successful. Virus was isolated from only 2 out of 17 samples and these isolates grew to low HA titres. Similar results have been reported by other workers (Stott et al, 1976; Bridger et al, 1978b). In our studies this may have been because the foetuses were obtained from an abattoir: there was a considerable time lapse between death of the foetuses and initiation of the cultures. The neonatal calves were available on site and thus delays were avoided. An alternative explanation is that the calf epithelial cells were more mature. Conditions in these cultures were therefore more similar to those present in the tracheas of young calves in which the virus multiplies naturally (McNulty et al, 1984).

An overall isolation rate of 48% was achieved in cultures derived from neonatal calves. An 100% success rate was highly unlikely, due to a number of factors. Firstly the ELISA used to detect BCV in faecal samples would also detect non-viable viruses and virus proteins. As BCVs are relatively fragile, some loss of viral infectivity was likely to occur during transport and storage of samples. Specific Abs present in the faeces are likely to bind the virus and render it non-infectious, although in some instances low concentrations of specific antiviral Ab can actually enhance virus growth (Peiris and Porterfield, 1981; Spaan et al, 1990). Finally, some enteric BCV strains may be incapable of growing in respiratory tract material. An alternative approach would have been to use intestinal organ cultures. Bridger et al (1978a) found that viruses originally isolated in TOC grew to higher HA titres in intestinal organ culture.

The ages of the calves from which the tracheas were taken had no apparent effect on the isolation rates of BCV. The SN titres may be more important: initial screening of calves to select those with low Ab titres might be worthwhile in future. The level of serum neutralising Ab is relevant in older calves at least, as the presence of serum neutralising Abs in these animals indicates previous exposure to the virus, which is likely to be accompanied by an immune response in the respiratory tract.

Adaptation of BCVs to growth in HRT-18 cells

Seven out of 12 viruses (58%) isolated in TOC were adapted to growth in HRT-18 cells. A higher success rate might have been achieved if some of the viruses had been passaged further. The success of virus adaptation may be related to the amount of infectious virus present in the original inoculum. All the viruses inoculated onto HRT-18 cells had HA titres of 64 or 128, but HA titres are not directly related to infectivity titres (Sato *et al*, 1977). It is possible that strain variation may also have affected the ability of some of the viruses to grow in HRT-18 cells. A large number of other cell lines have been used for *in vitro* growth of BCV (Laporte *et al*, 1979; Dea *et al*, 1980a; El-Ghorr, 1988). Use of these or different growth conditions might have permitted the adaptation of more viruses to growth in cell culture.

Growth curves: S2 strain BCV in HRT-18 cells

The growth curves obtained for S2 strain in HRT-18 cells were similar to those previously described for M strain grown in BEK

cells, S2 and F15 strains grown in HRT-18 cells and L-9 strain grown in BFTy cells (Sato et al, 1977; Laporte et al, 1980; Storz et al, 1981a; El-Ghorr, 1988; Keck et al, 1988). The HA titres started to rise rather later than the ITs. This was probably because HA tests lack the sensitivity required to detect the small amounts of virus released during the early stages of infection. A similar delay was observed for HCV OC38 grown in baby mice brains (Kaye and Dowdle, 1969) and BCV grown in BEK-1 cells (Sato et al, 1977). The HA titres continued to rise after ITs had peaked. This was probably due to release of non-infectious virions and subviral components in the later stages of infection. The observation that the distribution of HA activity in fractions collected after sucrose density gradient centrifugation is not identical to the distribution of virus infectivity supports the proposal that non-infectious virions and subviral components may have HA activity (Sharpee et al, 1976; Takahashi et al, 1983).

The growth curve constructed using an moi of 0.5 demonstrates that the optimal time to harvest inoculated virus is 48-72 h pi. To achieve optimal incorporation of radiolabel into virus proteins, the radiolabel should be added at 5 h pi, shortly before production of infectious virus particles commences at 6-9 h pi.

CHAPTER 4

MONOCLONAL ANTIBODIES TO BOVINE CORONAVIRUS

CHAPTER 4
MONOCLONAL ANTIBODIES TO BOVINE CORONAVIRUS

Introduction

Kohler and Milstein (1975) were the first workers to describe the production of MAbs using hybridoma technology. They successfully fused mouse myeloma cells with mouse spleen cells which had been harvested from an immunised donor. The resulting hybrid cells retained the ability of the myeloma cells to grow *in vitro* and secreted Abs directed against the original antigen used to immunise the spleen donor mouse. MAbs are now invaluable research tools in a variety of scientific disciplines. In virology they offer important advantages over conventional polyclonal or monospecific antisera. Their unique specificities make them particularly suitable for analysing virus proteins and for detecting antigenic variations between different strains of the same virus. Large quantities of homogeneous Abs can be obtained at high concentrations from hybridoma cell supernates or ascitic fluids.

Several groups have raised MAbs against BCV and used them to study the virus proteins (Deregt *et al*, 1983; Vautherot and Laporte, 1983; Deregt and Babiuk, 1987; Czerny and Eichhorn, 1989). The epitopes on the S and HE gps have been partially mapped (Deregt and Babiuk, 1987; Deregt *et al*, 1989b) and preliminary *in vivo* protection studies performed (Deregt *et al*, 1989a). MAbs have been used to improve the sensitivity and specificity of diagnostic tests for BCV (Crouch *et al*, 1984; Czerny and Eichhorn, 1989) and to detect antigenic variations between different virus isolates

(Vautherot and Laporte, 1983; Deregt et al, 1989a; El-Ghorr et al, 1989; Czerny and Eichhorn, 1989).

A panel of 8 MAbs was raised against S2 strain BCV (S2 MAbs) and a further 4 MAbs were supplied from the CVL, Weybridge (CVL MAbs). The MAbs were characterised in terms of their isotypes, protein specificities by Western blotting, and reactions with S2 strain BCV in IF, SN and HAI tests. They were later used in epitope mapping studies (Chapter 5), to look for antigenic variations between different isolates of the virus (Chapter 6) and to study the immunological response to individual viral proteins (Chapter 7).

Results

Production of MAbs

Spleen cells from mice immunised with S2 strain BCV were fused with NS-0 cells by Miss I. Campbell at MRI. Two separate fusions were performed. The hybridoma cells produced were screened for production of BCV specific Abs in IF, SN and HAI tests, and attempts made to clone the Ab positive cells 3 times by terminal dilution. The candidate tried to clone 11 of these cell lines, but 7 rapidly ceased to produce BCV specific Abs after the first cloning and a further cell line ceased Ab production after the third cloning. The remaining 3 hybridoma cell lines were successfully cloned 3 times and the MAbs they produced designated S2/4, S2/5 and S2/6. A further 5 hybridoma cell lines were cloned 3 times by Miss I. Campbell, and the MAbs produced by these cells were named S2/1, S2/2, S2/3, S2/7 and S2/8.

Cloned hybridoma cells were inoculated via the IP route into pristane-primed mice for ascitic fluid production. A batch of about 10 mice was injected with cells from each hybridoma. Harvested ascitic fluids were clarified and tested in the 3 screening tests. BCV Ab positive harvests were pooled, aliquoted and stored at -70°C. The hybridoma cell lines secreting MAbs S2/4, S2/7 and S2/8 produced relatively small volumes of ascitic fluids so these cells were inoculated into second groups of mice. The ascitic fluids containing MAbs S2/1, S2/2 and S2/3 were produced by Miss I. Campbell.

Isotypes

The isotypes of the MAbs produced in vitro were investigated. All 8 MAbs were first tested using a kit based on the Ouchterlony immunodiffusion technique (Binding Site kit). Test samples were pipetted into the central wells of a gel and the 6 surrounding wells received antisera specific to the different mouse heavy chain classes or subclasses (typing antisera). The gels were held at room temperature overnight and examined by the naked eye for the presence of precipitation arcs. The positive control serum gave precipitation lines with all 6 typing antisera. No precipitation lines were produced by negative control RPMI media supplemented with 5% FBS. The 8 test MAbs all gave a single precipitation line with the typing antisera which corresponded to their isotype (Table 4.1).

To increase the sensitivity of the test, the gels were stained with Coomassie Brilliant Blue. This resulted in clear staining of

Table 4.1

Determination of MAb isotypes

MAb	MAb isotypes, as determined by :		
	Binding Site kit		
	Unstained gel	Stained gel	Amersham kit
S2/1	IgG2a	IgG2a	NT
S2/2	IgG2a	IgG2a	IgG2a
		IgM	IgM*
S2/3	IgG2b	IgG2b	IgG2b
		IgM	IgM
S2/4	IgG2b	IgG2b	NT
S2/5	IgG2a	IgG2a	NT
S2/6	IgG2a	IgG2a	IgG2a
		IgG2b*	
S2/7	IgG2a	IgG2a	NT
S2/8	IgG1	IgG1	NT

NT = not tested.

* Pale band only.

the bands previously visible with the naked eye. Weaker reactions were also seen between MAbs S2/2, S2/3 and S2/6 and a second typing antiserum (Fig. 4.1, Table 4.1).

The 3 MAbs which reacted with 2 of the typing antisera were tested again using the Binding Site kit. This time the reacting antisera were each put into 2 adjacent wells around the central test well. After incubation and staining, lines of identity were seen between wells containing the same typing antisera. Lines of non-identity were seen between adjacent wells containing different typing antisera. This demonstrated that the 2 precipitation lines seen in the original tests were not due to reactions between identical antigens and Abs. Lines of identity would have indicated that the typing antisera were not specific for a single class or subclass of mouse heavy chain (Fig. 4.2).

These 3 MAbs were then tested using a kit based on the ELISA principle (Amersham kit). The results obtained for MAbs S2/2 and S2/3 were identical to those previously obtained with the Binding Site kit after staining with Coomassie Brilliant Blue. MAb S2/6 reacted with a single typing antiserum, the isotype corresponding to the strong precipitation line seen with the Binding Site kit (Fig. 4.3, Table 4.1).

Protein specificities

Binding of anti-mouse HRP conjugate to the MAbs

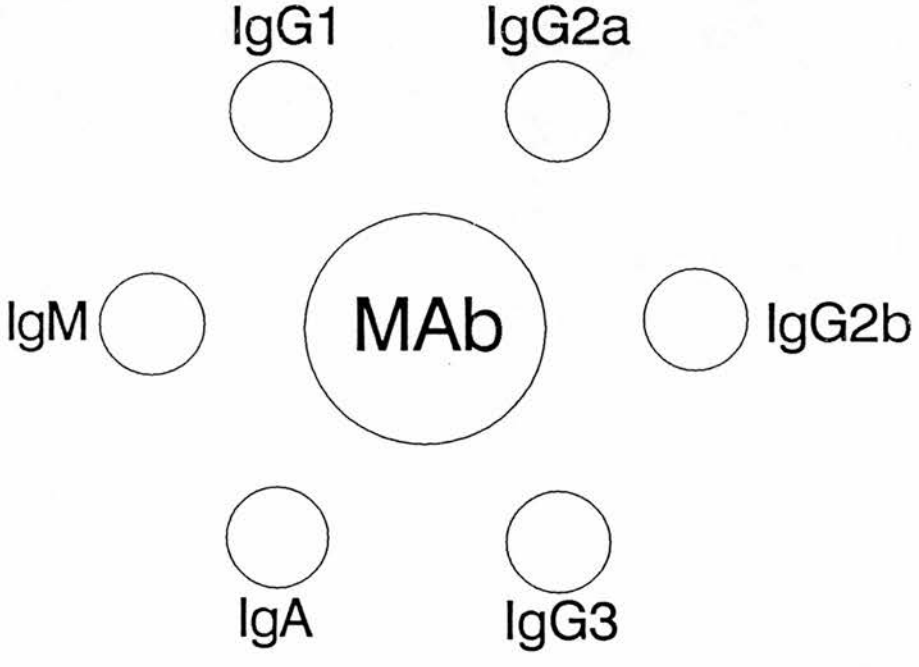
Anti-mouse HRP conjugate was used to detect MAbs bound to blotted viral proteins in some of the Western blotting experiments. A preliminary test was therefore performed to check that the

Figure 4.1

Determination of MAb isotypes : Binding Site kit

The S2 MAbs were tested with an isotyping kit produced by Binding Site, and the gel stained with Coomassie Brilliant Blue.

Template



+ and - indicates positive (positive control serum) and negative (RPMI/5%FBS) controls.

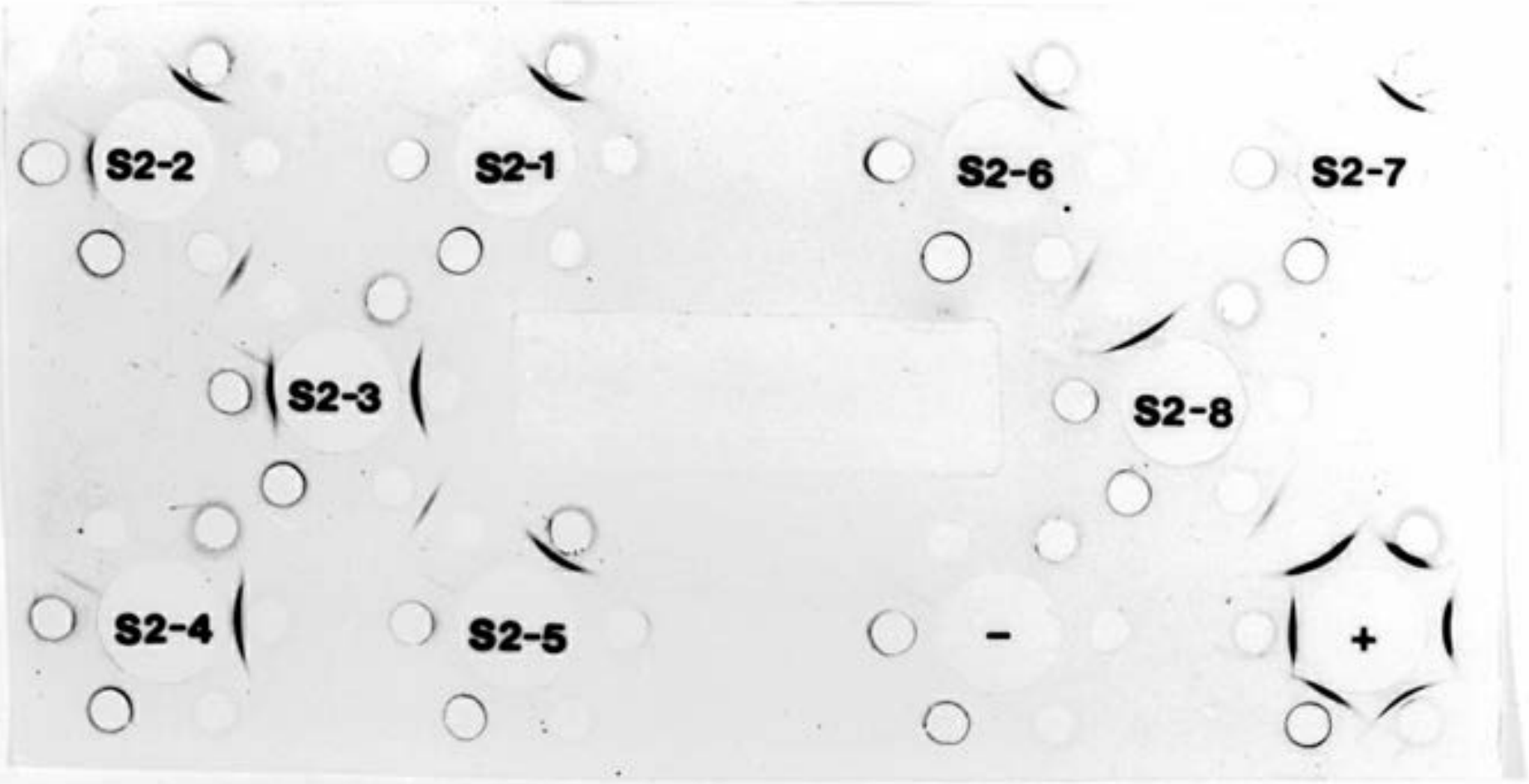
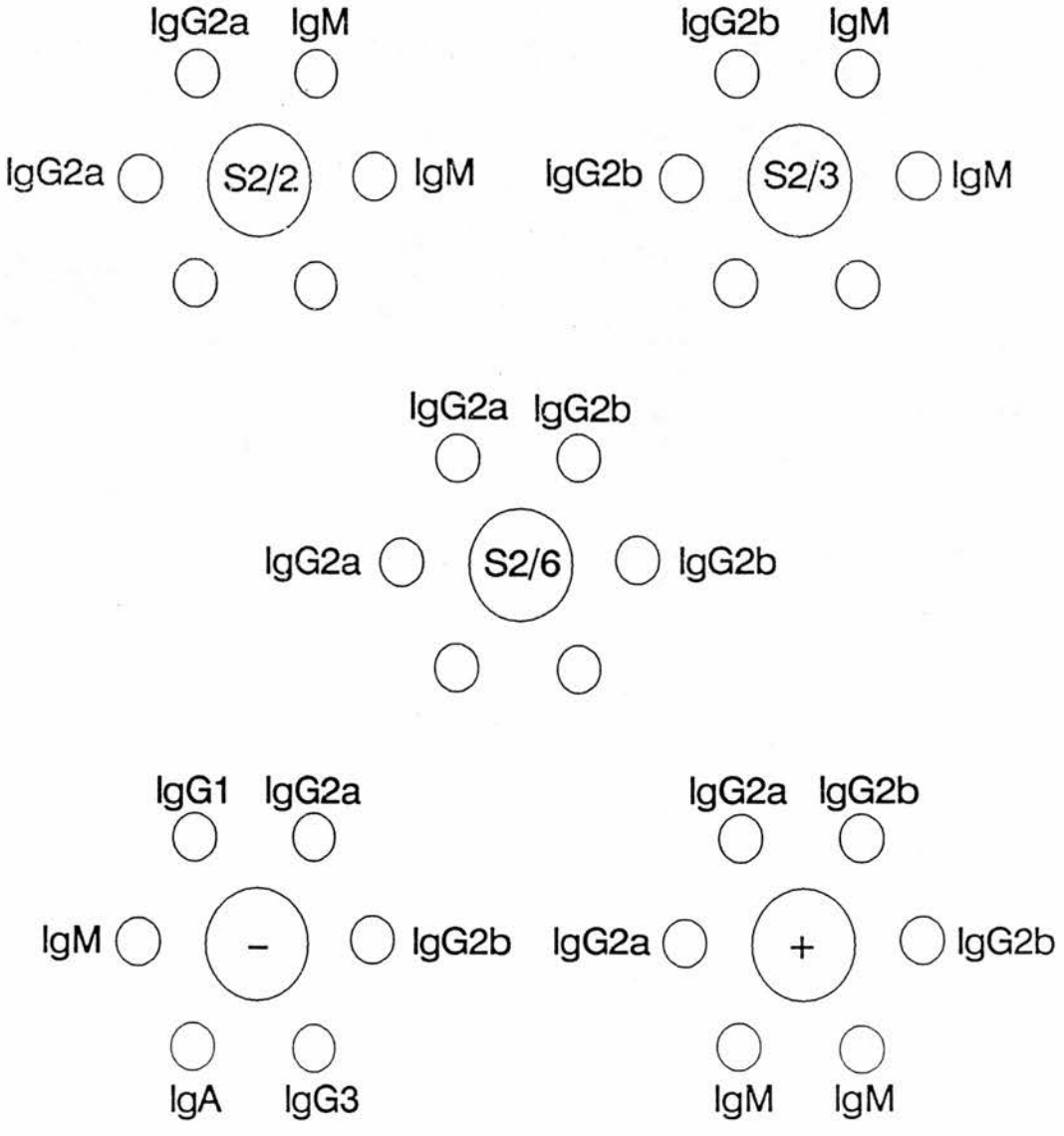

S2-2
S2-1
S2-6
S2-7
S2-3
S2-8
S2-4
S2-5
–
+

Figure 4.2

Determination of MAb S2/2, S2/3 and S2/6 isotypes : Binding Site kit

The MAbs S2/2, S2/3 and S2/6 were tested with an isotyping kit produced by Binding Site, and the gel stained with Coomassie Brilliant Blue.

Template



+ and - indicates positive (positive control serum) and negative (RPMI/5%FBS) controls.

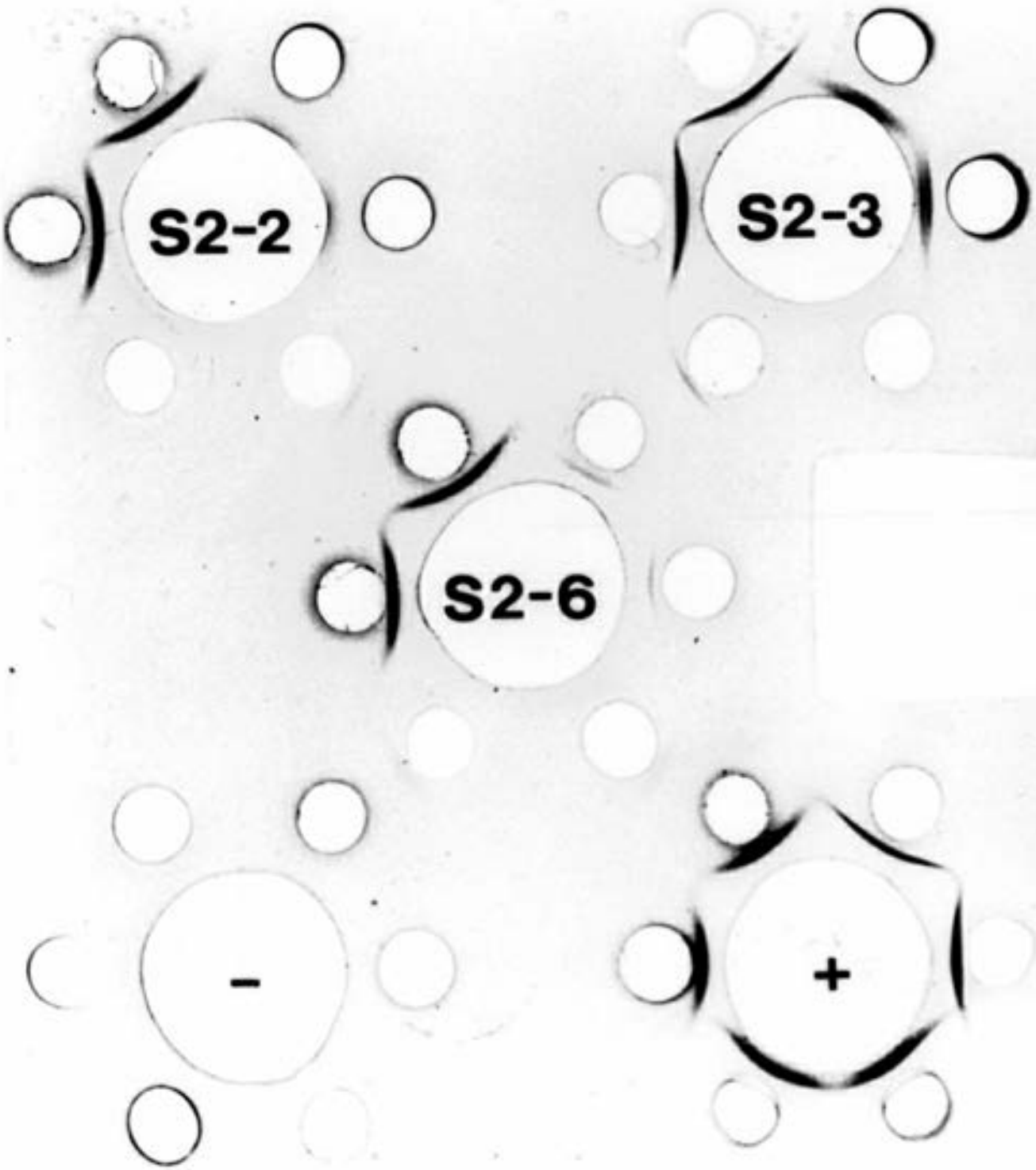
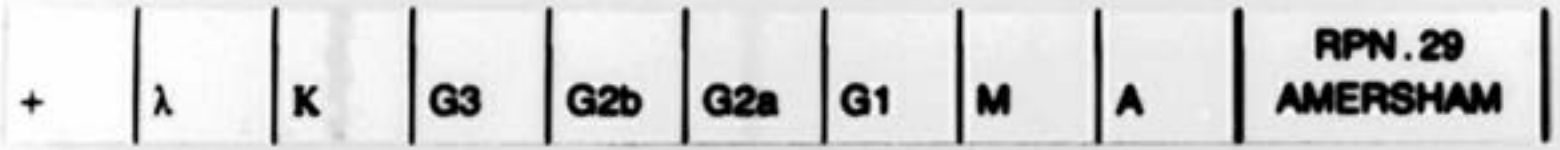

S2-2
S2-3
S2-6
-
+

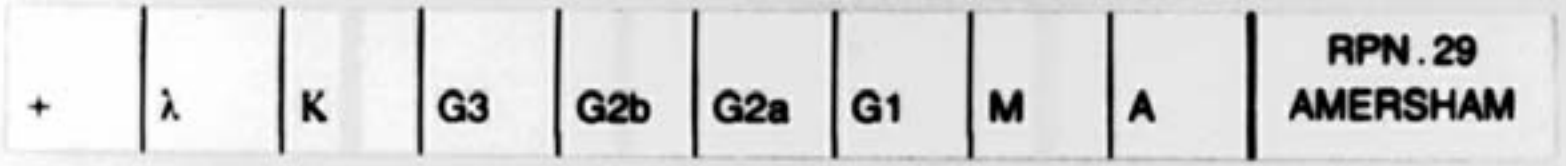
Figure 4.3

Determination of MAb S2/2, S2/3 and S2/6 isotypes: Amersham kit

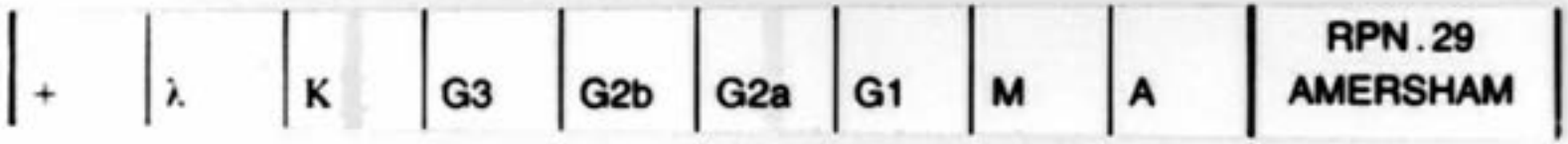
The MAbs S2/2, S2/3 and S2/6 were tested with an isotyping kit produced by Amersham.


+
λ
K
G3
G2b
G2a
G1
M
A
RPN.29
AMERSHAM

S2-2


+
λ
K
G3
G2b
G2a
G1
M
A
RPN.29
AMERSHAM

S2-3


+
λ
K
G3
G2b
G2a
G1
M
A
RPN.29
AMERSHAM

S2-6

conjugate bound to all the MAbs. 5 ul volumes of the S2 strain MAbs were spotted onto nitrocellulose paper at dilutions of 1:10, 1:100, 1:1000 and 1:10,000, and the paper soaked in PBS/0.5%T to block unbound protein binding sites. Bound MAbs were detected with anti-mouse HRP conjugate used at a previously determined optimal dilution of 1:100, followed by 4-chloro-1-naphthol substrate solution. The conjugate gave a moderate or strong reaction with all the MAbs when they were used at dilutions of 1:10 or 1:100 and a weak reaction with MAbs at dilutions of 1:1000. These results demonstrated that the conjugate was suitable for detecting bound MAbs in Western blotting experiments (Fig. 4.4).

Western blotting

The protein specificities of the MAbs were investigated by Western blotting. Three virus pellets were prepared from S2 strain BCV grown in HRT-18 cells by freeze/thawing the infected cultures, clarifying the resulting harvests and concentrating the virus by ultracentrifugation through sucrose cushions. The infectivity (IT) and HA titres of the virus pellets are summarised in Table 4.2. The virus proteins were dissociated by boiling with LSB containing SDS at a final concentration of 1%. 2-Mercaptoethanol (ME) was included at a final concentration of 2% in some experiments (reducing conditions). The proteins were separated by PAGE using 3% stacking and 10% resolving gels and blotted overnight onto 0.2um pore size nitrocellulose paper. The gels were stained with Coomassie Brilliant Blue and the nitrocellulose sheets stained with Ponceau

Figure 4.4

Binding of anti-mouse HRP conjugate to the MAbs

MAbs were spotted onto nitrocellulose paper at various dilutions and incubated with anti-mouse HRP conjugate at a dilution of 1:100. Bound conjugate was detected with 4-chloro-1-naphthol substrate solution.

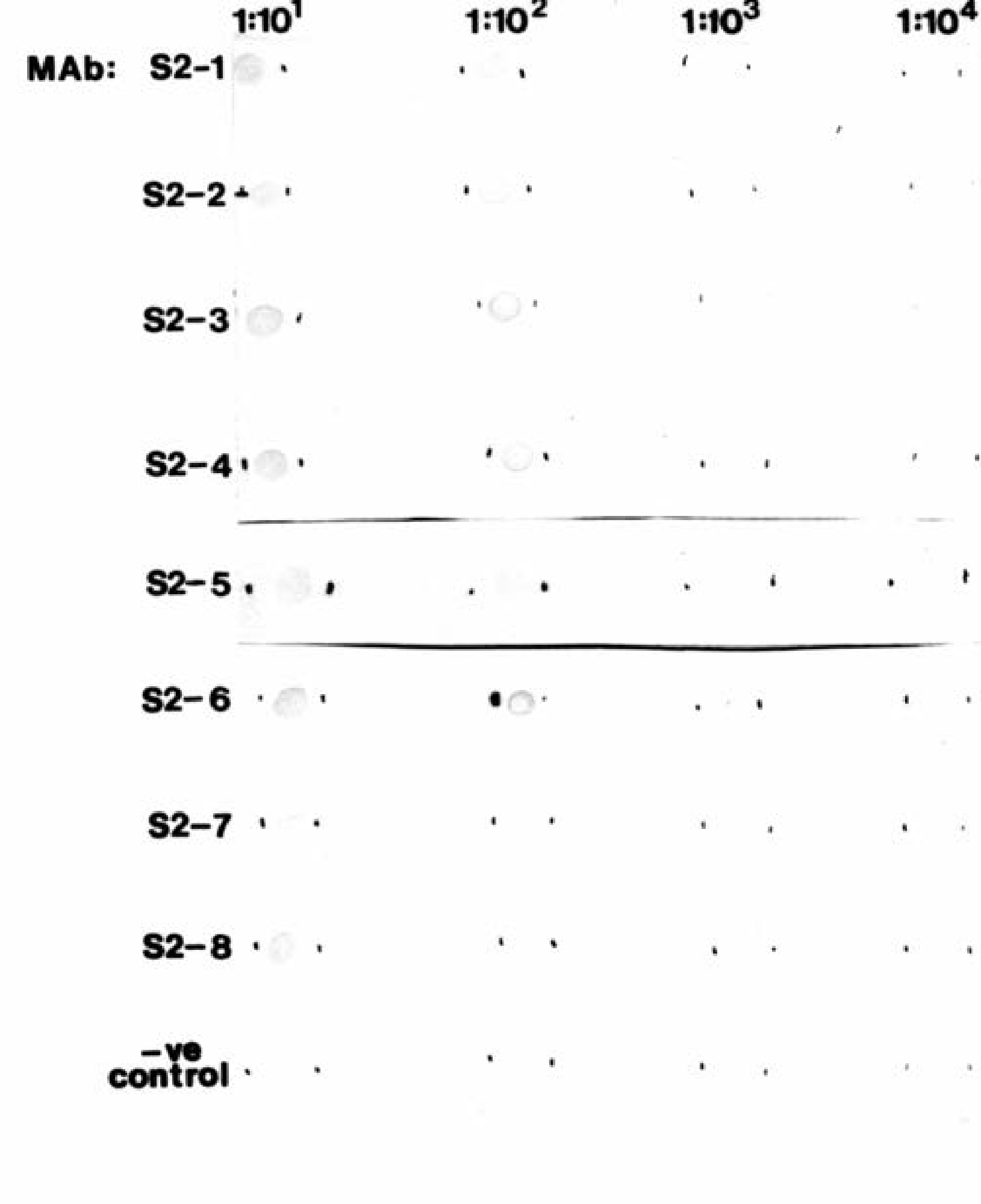

1:10^1
1:10^2
1:10^3
1:10^4
MAb:
S2-1
S2-2
S2-3
S2-4
S2-5
S2-6
S2-7
S2-8
-ve control

Table 4.2

Virus preparations used for Western blotting

Conditions	Detection system	Virus preparations : Protein estimation ug/ml	IT	HA titre
R	I-125	2350	6.8	3200
NR	I-125	2750	8.8	16000
R	HRP	1850	8.8	163840
NR	HRP	1850	8.8	163840

R = reducing NR = non-reducing

I-125 = I-125 protein A HRP = anti - mouse HRP conjugate

IT = log TCID50/ml

S. A few very pale bands were seen on the stained gels whilst many darkly stained bands were seen on the nitrocellulose paper. This demonstrated that the proteins had transferred efficiently to the nitrocellulose. The remaining protein binding sites on the nitrocellulose paper were quenched with PBS/0.5%T. The virus proteins were probed with MAbs present in diluted mouse ascitic fluids, the test dilutions being chosen to obtain the maximum feasible concentrations of MAbs for the test system being used. Bound MAbs were detected with ^{125}I protein A or with anti-mouse HRP conjugate. The MWs of stained bands were calculated by comparison with the migration patterns of standard MW markers. The identities of these bands were deduced from comparison with the published MWs of BCV proteins (Vautherot and Laporte, 1983; Deregt et al, 1987). The MWs of S2 strain proteins were later confirmed using polyclonal sera in Western blotting and RIP experiments (Chapter 5).

In the early experiments the nitrocellulose sheets bearing the blotted proteins were cut into strips. The strips were placed in tubes and probed with S2 MAb ascitic fluids at dilutions of 1:40. Bound MAbs were detected with ^{125}I protein A and the autoradiographs developed after 3 days. MAbs S2/5 and S2/8 were the only MAbs which bound to blotted proteins under reducing conditions. They both bound to a protein with a MW of about 56 KD, which corresponds to the MW of the N protein (Fig. 4.5, Table 4.3). Attempts to renature the blotted proteins by overnight incubation of the nitrocellulose sheet in PBS at 37^{o}C failed to result in the binding of any more of the MAbs. Blotting was also performed in the absence of ME (non-reducing conditions). Autoradiographs

Figure 4.5

MAb protein specificities determined by Western blotting (reducing conditions)

The proteins of S2 strain BCV were separated by SDS/PAGE, blotted onto nitrocellulose paper and probed with the MAbs. Bound MAbs were detected with ^{125}I protein A.

Template

Lane		Probe
1	S2 MAbs:	S2/1
2		S2/2
3		S2/3
4		S2/4
5		S2/5
6		S2/6
7		S2/7
8		S2/8
9	CVL MAbs:	5528
10		5529
11		5530
12		5531
13	+ve control:	Rabbit anti-S2 serum (serum 5317)
14	-ve control:	PBS/0.5%T

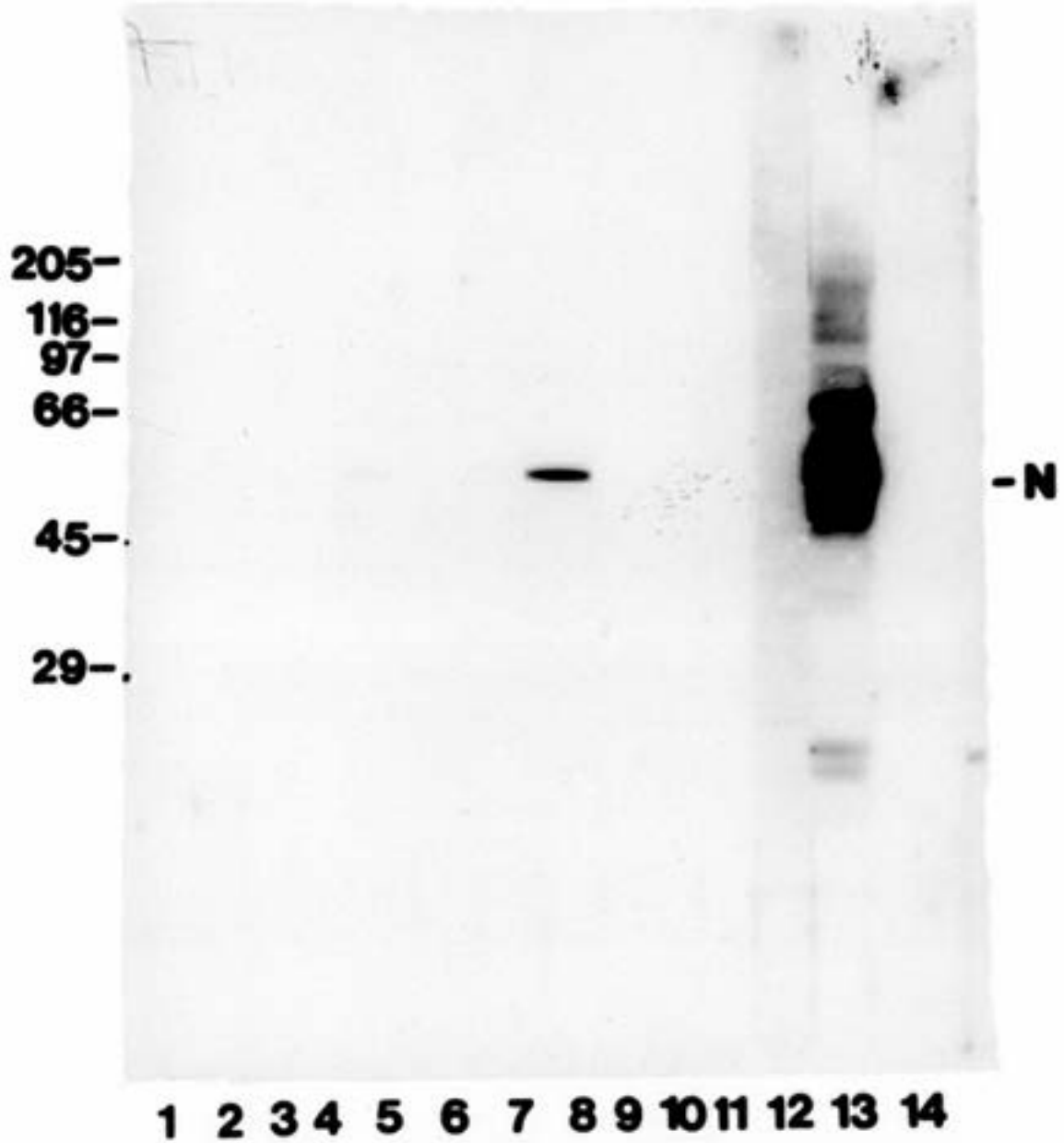

205-
116-
97-
66-
45-
29-
-N
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Table 4.3

Protein specificities of the MAbs determined by Western blotting

MAbs	Detection system used to detect protein bound MAbs :			
	I - 125 protein A		Anti - mouse HRP conjugate	
	Reducing*	Non - reducing*	Reducing*	Non - reducing*
S2 MAbs				
S2/1	-	-	-	-
S2/2	-	S	-	S
S2/3	-	HE	-	HE
S2/4	-	HE	-	HE
S2/5	N	N	N	N
S2/6	-	-	N	N
S2/7	-	-	-	HE
S2/8	N	N	N	N
CVL MAbs				
5528	-	-	/	M
5529	-	-	/	S
5530	-	-	/	S
5531	-	-	/	HE
MAb dilutions	S2 MAbs = 1:40 CVL MAbs = 1:1000	S2 MAbs = 1:40 CVL MAbs = 1:200	S2 MAbs = 1:4	S2 MAbs = 1:4 CVL MAbs = 1:10

* Virus proteins separated by SDS/PAGE under reducing or non-reducing conditions.

- = no reaction / = not tested

developed after 32 days revealed the binding of a further 3 MAbs to blotted proteins. MAb S2/2 bound to a 97 KD MW protein (S) and MAbs S2/3 and S2/4 bound to an 116 KD MW protein (HE) (Fig 4.6, Table 4.3). The CVL MAbs were used at dilutions of 1:1000 to probe reduced virus proteins and at dilutions of 1:200 to probe non-reduced virus proteins, the concentrations being increased in an attempt to obtain positive reactions. Binding of these MAbs to the blotted viral proteins was never observed under either set of conditions (Fig. 4.5 and 4.6, Table 4.3).

In subsequent experiments an Immunetics Miniblotter 25 was available for use when probing the blotted proteins. This allowed the ascitic fluids to be tested at much higher concentrations because smaller probe volumes were required. Ascitic fluids containing S2 MAbs were tested at dilutions of 1:4 and bound MAbs detected with an anti-mouse HRP conjugate. MAbs S2/5, S2/6 and S2/8 bound to the N protein (52 KD) under both reducing and non-reducing conditions. MAbs S2/2, S2/3, S2/4 and S2/7 only bound to blotted proteins under non-reducing conditions. MAb S2/2 bound to the S gp (98 KD) and MAbs S2/3, S2/4 and S2/7 bound to the HE gp (116 KD). MAb S2/1 failed to bind to blotted proteins even when neat ascitic fluid was tested. The CVL MAbs were tested at dilutions of 1:10 under non-reducing conditions only. 5528 bound to the M gps (18-22 KD), 5529 and 5530 bound to the S gp (102 KD) and 5531 bound weakly to the HE gp (116 KD) (Figs 4.7, 4.8 and 4.9, Table 4.3).

PBS/0.5%T, rabbit anti-rotavirus serum (1776) and ascitic fluid containing rotavirus MAbs (5328) were used as negative

Figure 4.6

MAb protein specificities determined by Western blotting (non-reducing conditions)

The proteins of S2 strain BCV were separated by SDS/PAGE, blotted onto nitrocellulose paper and probed with the MAbs. Bound MAbs were detected with ^{125}I protein A.

Template

Lane		Probe
1	S2 MAbs:	S2/1
2		S2/2
3		S2/3
4		S2/4
5		S2/5
6		S2/6
7		S2/7
8		S2/8
9	CVL MAbs:	5528
10		5529
11		5530
12		5531
13	+ve controls:	Rabbit anti-S2 serum (serum 5317)
14		Cow field serum (serum 5685)
15	-ve controls:	Rotavirus MAb (mouse ascitic fluid) (5328)
16		Rabbit anti-rotavirus serum (serum 1776)
17		PBS/0.5%T

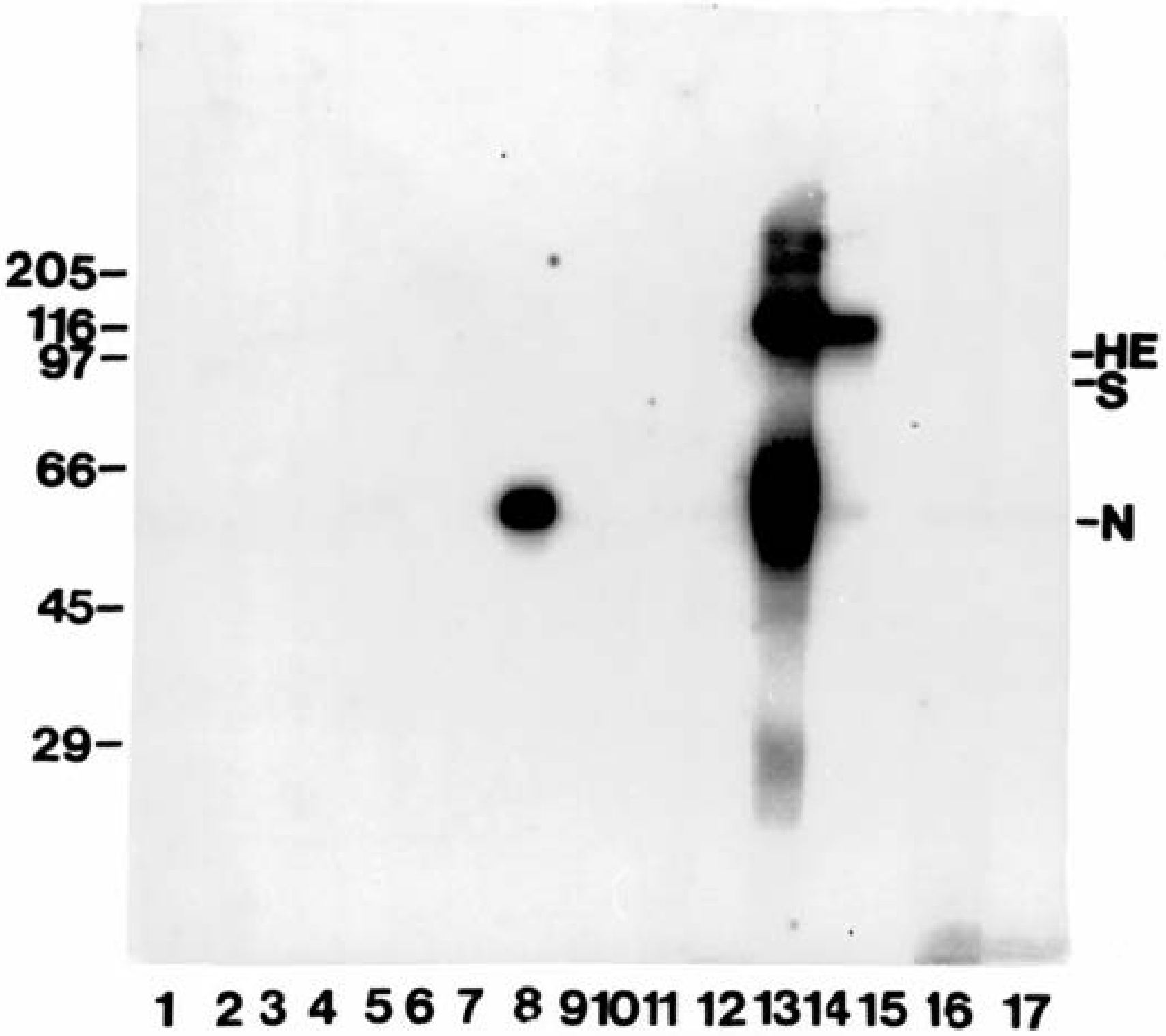

205-
116-
97-
66-
45-
29-
-HE
-S
-N
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 4.7

MAb protein specificities determined by Western blotting (reducing conditions)

The proteins of S2 strain BCV were separated by SDS/PAGE, blotted onto nitrocellulose paper and probed with the MAbs. Bound MAbs were detected with anti-mouse HRP conjugate.

Template

Lane		Probe
1	S2 MAbs:	S2/1
2		S2/2
3		S2/3
4		S2/4
5		S2/5
6		S2/6
7		S2/7
8		S2/8
9	-ve controls:	Rotavirus MAb (mouse ascitic fluid) (5328)
10		PBS/0.5%T

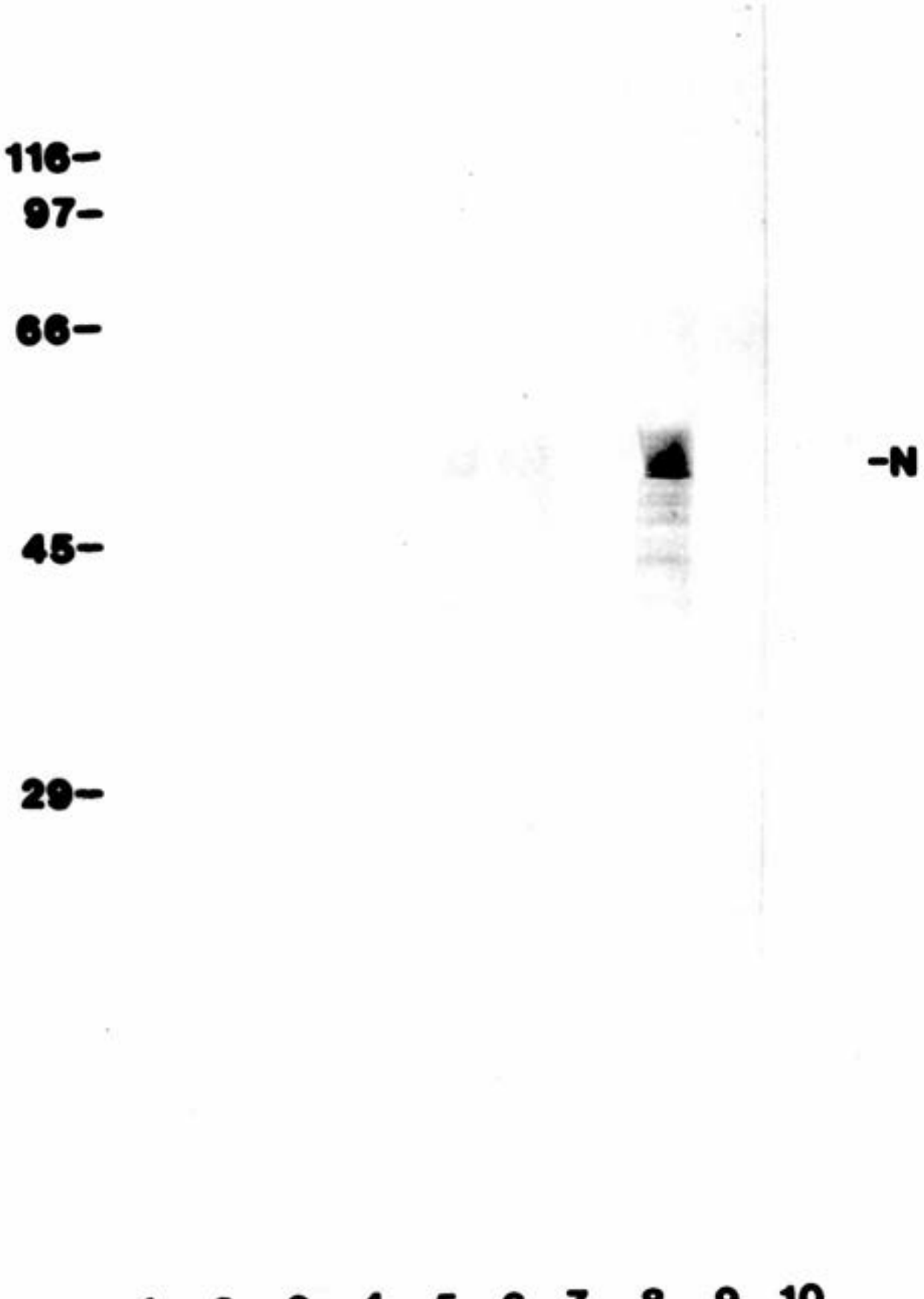

116-
97-
66-
45-
29-
-N
1 2 3 4 5 6 7 8 9 10

Figure 4.8

MAb protein specificities determined by Western blotting (non-reducing conditions)

The proteins of S2 strain BCV were separated by SDS/PAGE, blotted onto nitrocellulose paper and probed with the MAbs. Bound MAbs were detected with anti-mouse HRP conjugate.

Template

Lane		Probe
1	S2 MAbs:	S2/1
2		S2/2
3		S2/3
4		S2/4
5		S2/5
6		S2/6
7		S2/7
8		S2/8
9	-ve controls:	Rotavirus MAb (mouse ascitic fluid) (5328)
10		PBS/0.5%T

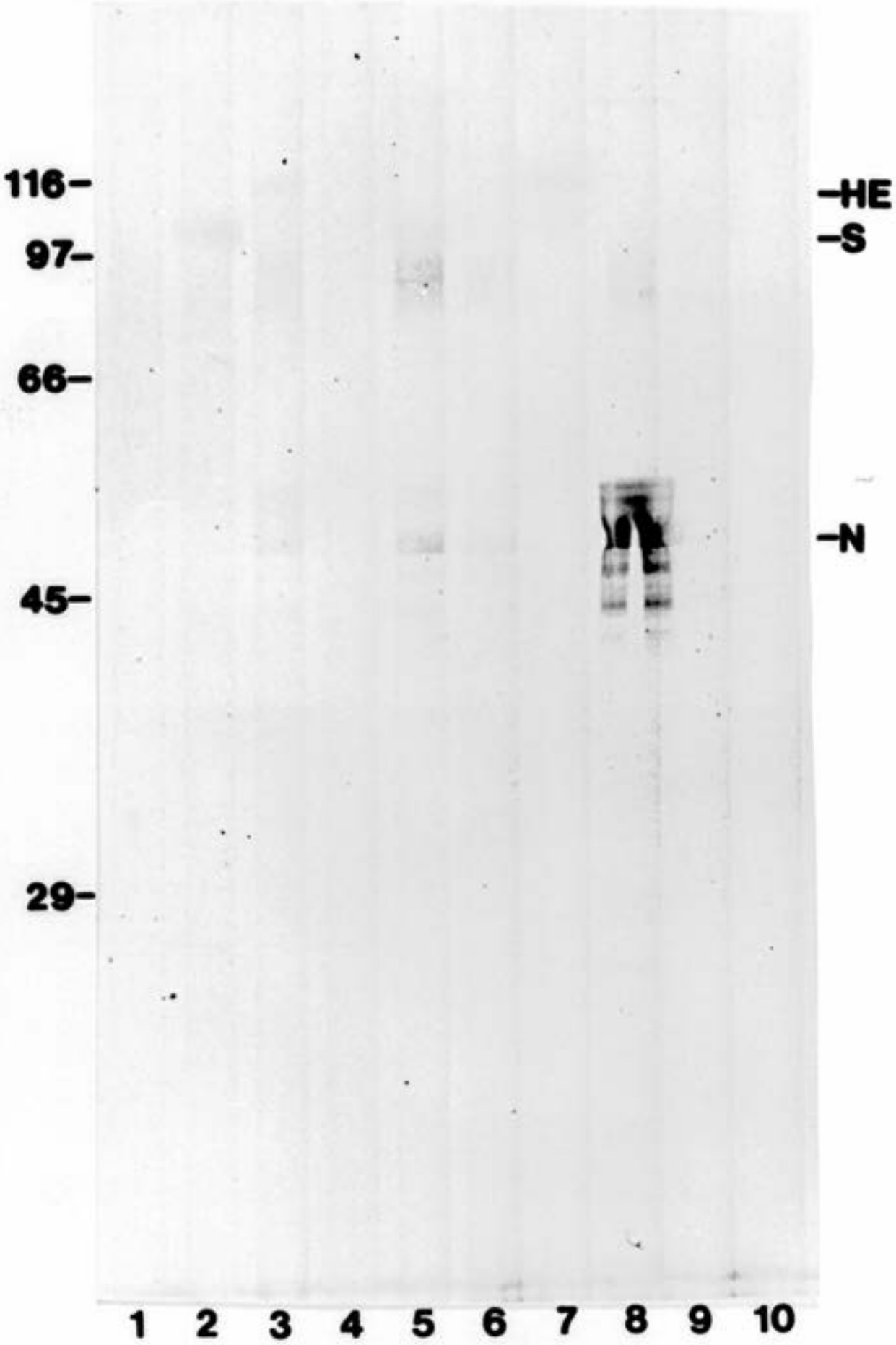

116–
97–
66–
45–
29–
–HE
–S
–N
1 2 3 4 5 6 7 8 9 10

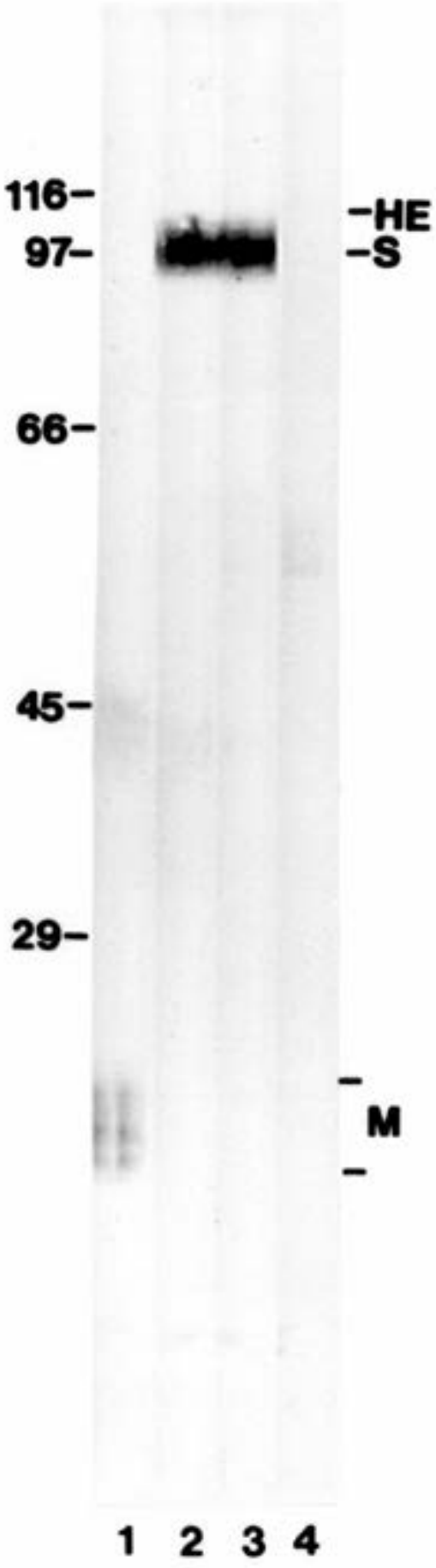
Figure 4.9

MAb protein specificities determined by Western blotting (non-reducing conditions)

The proteins of S2 strain BCV were separated by SDS/PAGE, blotted onto nitrocellulose paper and probed with the MAbs. Bound MAbs were detected with anti-mouse HRP conjugate.

Template

Lane		Probe
1	CVL MAbs:	5528
2		5529
3		5530
4		5531


116-
97-
66-
45-
29-
-HE
-S
M
1 2 3 4

control probes in the Western blotting experiments. Faint bands were sometimes observed in the N protein MW region when either these negative control probes or the test samples were used to stain blotted viral or MI HRT-18 cell proteins. They occurred using both ^{125}I protein A and anti-mouse HRP conjugate as the detection systems. Binding to blotted viral N proteins may be explained by the occurrence of non-specific interactions due to the unusual charge distribution on this protein. Similar non-specific binding has been reported between S and HE MAbs and the N protein of TCV (Dea and Tijssen, 1989a). The bands observed after probing MI HRT-18 cell proteins separated under reducing conditions may have been due to the presence of ME in the sample buffer during SDS/PAGE (Tasheva and Dessev, 1983; Herring and Sharp, 1984). A more likely explanation is that both ^{125}I protein A and anti-mouse HRP conjugate bind non-specifically to cellular proteins via hydrophobic interactions (Herring and Sharp, 1984; Birk and Koepsell, 1987). Binding of S2/5, S2/6 and S2/8 MAbs to blotted N proteins was clearly distinguishable from these non-specific reactions. These MAbs bound strongly to blotted viral proteins but failed to give a signal with blotted MI HRT-18 cell proteins.

A faint band at 83-94 KD MW was observed when many of the MAbs were used to probe virus proteins blotted under non-reducing conditions. The band was not observed with the negative control probes. It probably represented non-specific binding of MAbs to either viral or cellular proteins. The band was only seen when anti-mouse HRP conjugate was used as the detecting Ab, presumably because the higher concentrations of MAbs tested with this

conjugate lead to increased levels of non-specific binding (Birk and Koepsell, 1987).

Reactions in IF, SN and HAI tests

The MAbs were characterised in terms of their reactions with S2 strain BCV in IF, SN and HAI tests. Titres were expressed as the geometric means of duplicate results, rounded off to the nearest 10. Protein estimations were performed using a Pierce BCA Protein Assay kit.

These tests were first used to measure the titres of MAbs in unpurified ascitic fluids. The results obtained with MAbs raised against S2 and CVL strains of BCV are shown in Tables 4.4 and 4.5 respectively. The S2 MAbs were subsequently purified by affinity chromatography through a protein A sepharose column (Chapter 5). The results obtained using these purified ascitic fluids in IF, SN and HAI tests are shown in Table 4.6.

The titres obtained in these tests can be compared more easily if allowance is made for the variations in the Ig concentrations of the ascitic fluids. In purified ascitic fluids, total protein estimations roughly equate to MAb concentrations. The limiting concentrations of MAbs in purified ascitic fluids at the endpoint titres in IF, SN and HAI tests can be calculated from the titres and protein estimations given in Table 4.6, knowing that 50 ul of test samples were used in IF and HAI tests, and 25 ul in SN tests. These limiting concentrations are expressed in ug/ml in Table 4.7.

The N MAbs all reacted to low titres in IF tests and were negative in SN and HAI tests. The S MAb (S2/2) reacted to low

Table 4.4

BCV S2 MAbs

Unpurified ascitic fluids: IF, SN and HAI titres

BCV S2 MAbs

Unpurified ascitic fluids : IF, SN and HAI titres

Protein specificity	MAb	Titres* IF	SN	HAI	Protein estimation ug/ml
N	S2/5	9050	<100	<20	29
	S2/6	18100	<100	<20	40
	S2/8	2260	<100	<20	33
S	S2/2	4530	6400	320	29
HE	S2/1	102400	6400	5120	21
	S2/3	25600	1600	640	51
	S2/4	12800	200	3200	41
	S2/7	51200	6400	3200	33

* Titres are expressed as the geometric means of duplicate results, rounded off to the nearest 10.

Table 4.5

BCV CVL MAbs

Unpurified ascitic fluids: IF, SN and HAI titres

BCV CVL MAbs

Unpurified ascitic fluids : IF, SN and HAI titres

Protein specificity	MAb	Titres* IF	SN	HAI
M	5528	2260	<100	<60
S	5529	18100	<100	<60
	5530	18100	<100	<60
HE	5531	12800	100	<60

* Titres are expressed as the geometric means of duplicate results, rounded off to the nearest 10.

Table 4.6

BCV S2 MAbs

Purified ascitic fluids: IF, SN and HAI titres

BCV S2 MAbs

Purified ascitic fluids : IF, SN and HAI titres

Protein specificity	MAb	Titres* IF	SN	HAI	Protein estimation ug/ml
N	S2/5	200	<100	<50	1150
	S2/6	100	<100	<50	430
	S2/8	100	<100	<50	840
S	S2/2	400	400	<50	1690
HE	S2/1	6400	400	800	800
	S2/3	1600	140	<50	1030
	S2/4	1600	<100	130	720
	S2/7	6400	1130	800	1000

*Titres are expressed as the geometric means of duplicate results, rounded off to the nearest 10.

Table 4.7

BCV S2 MAbs

Purified ascitic fluids: Limiting IF, SN and HAI concentrations

BCV S2 MAbs

Purified ascitic fluids : Limiting IF, SN and HAI concentrations

Protein specificity	MAb	Limiting concentrations* in tests: IF	SN	HAI
N	S2/5	5.8	>11.5	>23.0
	S2/6	4.3	>4.3	>8.5
	S2/8	8.4	>8.4	>16.8
S	S2/2	4.2	4.2	>33.7
HE	S2/1	0.1	2.0	1.0
	S2/3	0.6	7.3	>20.5
	S2/4	0.5	>7.2	5.6
	S2/7	0.2	0.9	1.3

* Expressed as ug/ml at endpoints.

titres in IF and SN tests and occasionally gave a weak positive reaction in HAI tests. The 2 MAbs raised against the S gp of CVL strain BCV reacted strongly with S2 virus in IF tests and were negative in SN and HAI tests. The 4 MAbs raised against the HE gp of S2 strain BCV were positive in all 3 tests. MAbs S2/1 and S2/7 reacted strongly in all the tests whilst MAbs S2/3 and S2/4 reacted to high titres in the IF tests only. The MAb raised against the HE gp of CVL strain BCV reacted to a high titre in IF tests, a low titre in SN tests and was negative in HAI tests.

IF staining with MAbs

HRT-18 cells were grown on coverslips and infected with S2 virus. At 20 h pi some of the cultures were fixed with acetone and probed with the MAbs to study the patterns of IF staining. Other cultures were left unfixed and immediately probed with the MAbs to investigate their abilities to stain virus antigens on the membranes of infected cells. In both procedures, MAbs were tested at IF titres of 128, and bound MAbs detected with anti-mouse IgG FITC. The level of non-specific binding of this conjugate was ascertained by staining cells probed with PBS in place of the MAbs. No IF was observed when MI HRT-18 cells were probed with the MAbs.

The results obtained in these tests are summarised in Table 4.8, and the IF patterns demonstrated in Figs 4.10 to 4.15. The 3 N MAbs gave a fairly even distribution of IF staining throughout the cytoplasm of the cells. MAb S2/8 gave a particularly distinctive pattern as the staining was very even throughout the cytoplasm and cell boundaries were clearly demarcated. The IF staining produced

Table 4.8

IF staining with MAbs

IF staining with MAbs

Protein specificity	MAb	IF in fixed virus infected cells Intensity*	Distribution**	Pattern***	Membrane fluorescence
N	S2/5	+	C	G	-
	S2/6	+	C	G	-
	S2/8	++	C	LG	-
S	S2/2	++	P	G	+
HE	S2/1	++	P	G	+
	S2/3	++	C+P	G	+
	S2/4	++	C+P	LG	+
	S2/7	++	P	G	+

* + = moderate ++ = strong

** C =mainly cytoplasmic P = mainly perinuclear C+P = cytoplasmic and perinuclear

*** G = granular LG = large granules

Figure 4.10

Acetone fixed S2 infected HRT-18 cells probed with MAb S2/1

Figure 4.11

Acetone fixed S2 infected HRT-18 cells probed with MAb S2/2.

Figure 4.12

Acetone fixed S2 infected HRT-18 cells probed with MAb S2/3.

Figure 4.13

Acetone fixed S2 infected HRT-18 cells probed with MAb S2/4.

Figure 4.14

Acetone fixed S2 infected HRT-18 cells probed with MAb S2/6.

Figure 4.15

Acetone fixed S2 infected HRT-18 cells probed with MAb S2/8.

by the S and HE MAbs was concentrated in the perinuclear regions of the cells. All MAbs produced a granular pattern of IF staining: large distinctive granules were observed with MAbs S2/4 and S2/8. The S and HE MAbs stained the membranes of unfixed virus infected cells whilst membrane fluorescence was never observed with the N MAbs.

Discussion

Production of MAbs

Eight hybridoma cell lines were established which secreted MAbs directed against S2 strain BCV, and a further 4 MAbs directed against CVL strain of BCV were supplied from CVL, Weybridge.

Isotypes

It was important to determine the isotypes of the MAbs because this affected their behaviour and choice of reagents in subsequent experiments. Knowledge of their isotypes was particularly relevant when choosing the detection systems for Western blotting and IF tests and when purifying MAbs from ascitic fluids.

The heavy chains of 6 of the 8 MAbs were identified as single isotypes. MAbs S2/2 and S2/3 reacted weakly with second typing antisera in both the Binding Site and Amersham kits, possibly because the typing antisera were not monospecific. These antisera may have contained Abs to more than one mouse heavy chain isotype, although this did not appear to be true for the Binding Site kit because lines of non-identity were observed when the 2 typing antisera reacting with a single MAb were placed in adjacent wells.

The typing antisera may also have contained Abs to other proteins present in the hybridoma cell supernates. These proteins may be derived either from the media or from the cells. FBS is the most abundant of these proteins, but no reaction was observed when RPMI/5% FBS was used as a negative control. The cross reactions may have occurred because the test MAbs were at high concentrations. The reactions of S2/2 and S2/3 MAbs with the IgM typing antisera were abolished when Miss I. Campbell tested the hybridoma supernates in the Amersham kit at dilutions of 1:100 instead of 1:10.

It is conceivable that the hybridoma cells secreting MAbs S2/2 and S2/3 produced Abs of 2 different isotypes. This may occur if the cells have not been adequately cloned. Hybridoma cells secreting S2/2 MAbs were therefore cloned for a fourth time by Miss I. Campbell but 2 isotypes were still detected. An alternative explanation is that the hybridoma cells were undergoing a class switch (Koolen et al, 1984; Aguila et al, 1986).

The MAbs were later purified from ascitic fluids by affinity chromatography on a Protein A sepharose column. The active fractions were eluted off the column at the pHs corresponding to the expected isotypes of the MAbs. There was no evidence that 2 isotypes were present in any of the ascitic fluids. These data and the fact that similar problems occurred when typing bovine rotavirus MAbs suggest that the weak reactions of the MAbs with second typing antisera were due to lack of specificity of the typing antisera supplied with the test kits.

Protein specificities

The protein specificities of 11 out of the 12 MAbs were determined by Western blotting. Three of these MAbs (S2/5, S2/6 and S2/8) bound to the N protein under both reducing and non-reducing conditions. Probing of blotted viral proteins with these MAbs revealed a strongly staining broad band around the 52 KD MW region. A series of less strongly staining bands of slightly greater electrophoretic mobility was revealed when anti-mouse HRP conjugate was used to detect bound MAbs. Similar bands were seen when polyclonal sera were used to detect blotted proteins (Chapter 5). These bands were not observed when blotted MI HRT-18 cell proteins were probed with test Abs or when blotted S2 proteins were probed with negative control samples. This suggests that this series of bands is produced by specific binding of Abs to N protein precursors or degradation products. Similar bands have been detected by other groups using both polyclonal sera and MAbs (Deregt et al, 1983; Vautherot and Laporte, 1983; Keck et al, 1988; Dea and Tijssen, 1989a).

A single MAb was directed against the M gp (5528), 3 MAbs were directed against the S gp (S2/2, 5529 and 5530) and 4 MAbs were directed against the HE gp (S2/3, S2/4, S2/7 and 5531). These MAbs only bound to blotted proteins under non-reducing conditions, demonstrating that disulphide bonds are essential for the antigenic integrity of these epitopes. Deregt and Babiuk (1987) also found that the majority of MAbs only bind to blotted S and HE gps under non-reducing conditions.

MAb S2/1 failed to bind to blotted proteins, despite having a high binding affinity in ELISAs (Chapter 5). This suggests it is directed against a conformational epitope which is destroyed by SDS. Incubation of blotted proteins overnight in PBS at 37°C failed to renature the epitope. An alternative procedure would have been to use a gel system incorporating less denaturing detergents, but there would then have been a danger of incomplete protein separation (Birk and Koepsell, 1987). Results obtained in competition ELISAs (Chapter 5) strongly suggest that this MAb is directed against the HE gp.

The anti-mouse HRP conjugate detected the binding of 6 MAbs to virus proteins which were not detected using ^{125}I protein A. There are several possible explanations to account for this behaviour. Firstly, the HA titre of the virus pellet used with the anti-mouse HRP conjugate detection system was 10-fold greater than those used with ^{125}I protein A, facilitating the binding of the HE MAbs. Secondly, the S2 MAbs were tested at 10-fold higher concentrations with the anti-mouse HRP conjugate. Finally, the anti-mouse HRP conjugate may have been more sensitive than the ^{125}I protein A for detecting bound MAbs. The relative sensitivity of the 2 systems is dependent on both the initial binding of either anti-mouse HRP conjugate or protein A to the MAbs, and on their subsequent detection by reaction with substrate solution or by autoradiography respectively. In theory, anti-species specific conjugates are more sensitive than protein A for detecting bound Abs because several molecules bind to each Ig molecule, leading to signal amplification. In contrast, only 1 or 2 molecules of protein A

bind to each Ig molecule (Goding, 1978). Protein A also has the disadvantage that it is unsuitable for detecting certain isotypes. It binds strongly to mouse IgG2a and IgG2b but only weakly to IgG1 (Goding, 1978; Bjorck and Kronvall, 1984). This was not a problem in these tests as the strongest signal was produced by MAb S2/8 (IgG1 isotype). The enhanced sensitivity of the anti-species HRP conjugate caused by its favourable binding abilities with Igs is offset by the fact that detection by autoradiography is a more sensitive technique than enzymic reaction with a substrate solution (Towbin and Gordon, 1984).

The anti-species HRP conjugate probe proved to be sufficiently sensitive for these experiments. The sensitivity of the detection system could have been increased by using alkaline phosphatase or colloidal gold conjugated probes, or by amplifying the signal by incorporating an avidin-biotin or peroxidase-anti-peroxidase stage. The sensitivity of these experiments could have been improved further by replacing the nitrocellulose paper with a nylon based membrane such as Zetabind, which has a greater protein binding capacity than nitrocellulose (Gershoni and Palade, 1983; Towbin and Gordon, 1984).

Reactions in IF, SN and HAI tests

The reaction patterns of the MAbs in IF, SN and HAI tests were similar to those obtained by other groups working with BCV specific MAbs (Deregt et al, 1983; Vautherot et al, 1984; Deregt and Babiuk, 1987).

The N MAbs were positive in IF tests only, a result consistent with published reports. A few MAbs raised against the N proteins of other CVs are capable of in vitro virus neutralisation and in vivo protection (Buchmeier et al, 1984; Lecomte et al, 1987). The M MAb was also positive in IF tests only. Some MAbs directed against the M gps of other CVs can cause virus neutralisation, but complement may be required for this activity (Collins et al, 1982; Laude et al, 1986; Gilmore et al, 1987; Simkins et al, 1989).

The S and HE gps carry the main neutralising epitopes, but MAbs have also demonstrated the presence of non-neutralising epitopes on both these proteins (Vautherot et al, 1984; Dea and Tijssen, 1989a). In these experiments all 5 HE MAbs and only 1 out of 3 S MAbs neutralised S2 strain BCV. The ability of a MAb to neutralise the virus in vitro does not necessarily correlate with its ability to protect in vivo (Buchmeier et al, 1984; Talbot et al, 1984; Deregt et al, 1989a). Due to the lack of a suitable animal model it was not possible to determine whether these MAbs lead to in vivo protection.

MAbs directed against the HE gp of BCV vary in their ability to inhibit virus induced haemagglutination (Deregt et al, 1983; Parker et al, 1989). In these experiments only the HE MAb raised against CVL strain BCV failed to inhibit haemagglutination caused by S2 virus; this may have been due to lack of epitope conservation. MAb S2/2 also demonstrated weak HAI activity, which was surprising because this MAb is directed against the S gp. HAI activity has also been demonstrated with 2 MAbs directed against the S gp of G110 strain BCV (Vautherot et al, 1984), with 4 MAbs

directed against the S gp of L9 strain BCV (Storz et al, personal communication) and with a single MAb directed against the S gp of TCV (Dea and Tijssen, 1989a). The S gp may carry part or all of the virus receptor for RBCs and be directly involved with haemagglutination. An alternative explanation is that some epitopes on the S and HE gps are structurally or spatially closely related. The involvement of the S and HE gps in causing virus induced haemagglutination is discussed in more detail in Chapter 8.

The existence of MAbs with the same protein specificities but different reaction patterns demonstrates the presence of different antigenic regions. The MAbs raised against the S and HE gps of S2 strain BCV were all IF+ SN+ HAI+. The MAbs raised against the S and HE gps of CVL strain were IF+ SN- HAI- and IF+ SN+ HAI- respectively. These data suggest that there are at least 2 antigenic regions on each of these virus proteins. An alternative explanation is that the epitopes involved in SN and HAI are not conserved between the 2 viruses. Unfortunately the CVL strain virus was not available for testing this theory.

IF staining with MAbs

The patterns of IF staining produced by the MAbs correlated with their protein specificities. The N MAbs gave an even distribution of staining throughout the cell cytoplasm. The S and HE MAbs stained the perinuclear areas of the cells more intensely. These areas correspond to the positions of the Golgi and RER which are the sites where the S and HE proteins are glycosylated (Holmes, 1990). Similar patterns of staining have been demonstrated by MAbs

directed against TCV (Dea and Tijssen, 1989a) and TGEV (Laude et al, 1986; Welch and Saif, 1988).

Virus antigens were detected on the surface of infected cells only by MAbs directed against the S and HE gps. Similar results have been obtained using MAbs raised against TCV (Dea and Tijssen, 1989a), MHV (Collins et al, 1982; Wege et al, 1984) and TGEV (Laude et al, 1986; Welch and Saif, 1988). However membrane fluorescence has also been observed with a MAb directed against the N protein of MHV (Lecomte et al, 1987). Presence of virus proteins on the infected cell surface is unexpected because CVs mature in an intracellular location. It is explained by the transport of excess S and HE gps to the surface of infected cells (Kienzle et al, 1990; Parker et al, 1990). Here these proteins may facilitate spread of virus between cells by initiating membrane fusion events. They may also render the cell susceptible to attack by the host's defence system, allowing the elimination of infected cells before infectious virus particles are released. Destruction of these cells may be achieved by one of several mechanisms. Cytotoxic T lymphocytes and natural killer cells may lyse virus infected cells, either directly or in conjunction with virus-specific Abs (Ab-dependent cell-mediated cytotoxicity). Binding of Abs to virus antigens on the infected cell surface may also cause cell lysis by activation of the complement system, and facilitate uptake of the infected cells by macrophages.

CHAPTER 5

STRUCTURAL PROTEINS OF BOVINE CORONAVIRUS

CHAPTER 5
STRUCTURAL PROTEINS OF BOVINE CORONAVIRUS

Introduction

The structural proteins of BCV have been described by several groups (King and Brian, 1982; Vautherot and Laporte, 1983; Deregt et al, 1987), but the antigenic regions of these proteins have not yet been clearly defined.

Competition ELISAs have been used to study the S and HE gps of BCV (Deregt and Babiuk, 1987). These tests are based on the assumption that binding of a MAb to a specific site hinders the binding of a second MAb which recognises the same or an overlapping site. The areas defined by these tests should be referred to as antigenic regions rather than epitopes, as epitopes are defined as occurring on the surface of native proteins (Laver et al, 1990). Deregt and Babiuk (1987) defined 2 antigenic regions (A and B) on the S gp with 5 strongly neutralising MAbs. Analysis of fragments generated by proteolysis of Ag-Ab complexes revealed that both regions were situated on a 37 KD fragment (Deregt et al, 1989b). A further antigenic region was defined by a single weakly neutralising MAb. Four MAbs defined 3 antigenic regions (A, B and C) on the HE gp in competition ELISAs. Region B overlapped regions A and C, and region A could be subdivided into 2 areas, A1 and A2, on the basis of results obtained in RIP tests. Region A was defined by MAbs with high SN and HAI titres and regions B and C by MAbs with low or negative SN and HAI titres (Parker et al, 1989). MAbs directed against antigenic region A of both the S and HE gps protected in vivo whilst MAbs directed against region B of the S gp

and C of the HE gp failed to protect *in vivo*. Failure of the former MAb to protect *in vivo* may have been due to lack of epitope conservation on the challenge isolate (Deregt *et al*, 1989a). The ability of MAbs directed against region B of the HE gp to protect *in vivo* has not yet been investigated.

Further analysis of the structural proteins of BCV is required: the antigenic regions which elicit neutralising Abs are of particular interest. Viral antigens may be analysed on a functional or a structural basis and MAbs are ideal tools for these studies because of their unique specificities. A functional analysis may be performed by studying the reactions of a panel of MAbs with the parent virus in a series of serological tests (Chapter 4). Further information is gained by comparing the results obtained using different strains of the virus (Chapter 6). This chapter describes the characterisation of the proteins of S2 strain BCV in terms of their MWs in Western blotting and RIP experiments. MAbs raised against this virus were purified from ascitic fluids by affinity chromatography and their binding affinities assessed in ELISAs. They were then biotinylated and used in competition ELISAs to map the antigenic regions of the HE and N proteins. Methods of analysing antigens on a structural basis are discussed briefly at the end of this chapter.

Results

MWs of S2 viral proteins

The MWs of the structural proteins of S2 strain BCV were

determined by Western blotting and RIP. The former technique proved to be the more successful.

Western blotting

BCV specific Abs

The proteins of pelleted S2 strain BCV were dissociated by boiling with LSB in the presence or absence of ME, separated by SDS/PAGE and electroblotted onto nitrocellulose paper. The proteins were probed with a variety of polyclonal sera (at dilutions of 1:4) and MAbs at dilutions of 1:10 (MAb S2/8), 1:20 (CVL MAbs) and neat (S2/7 MAb). Negative control samples consisted of PBS/0.5%T, gnotobiotic lamb anti-MI HRT-18 cell sera (sera 5923 and 5926) and gnotobiotic lamb anti-rotavirus serum (serum 3626): the serum samples were used at dilutions of 1:4. Bound antibodies were detected with appropriate anti-species HRP conjugates used at previously determined optimal dilutions of 1:100, followed by 4-chloro-1-naphthol substrate solution. The MWs of the virus proteins were determined by comparison with the migration patterns of standard MW markers.

The results obtained using viral proteins separated under non-reducing conditions are shown in Fig. 5.1. The MWs of the HE, S, N and M proteins were calculated to be 116, 98, 52 (range 43 to 62) and 21 (range 19 to 23) KD respectively. In this figure an extra band with a MW of 98 KD is observed in the lane probed with MAb S2/7: this is probably an artefact caused by overflow of MAb 5529 from the adjacent lane.

Viral proteins were also separated under reducing conditions and probed with neat gnotobiotic calf anti-S2 serum (serum 5890) and

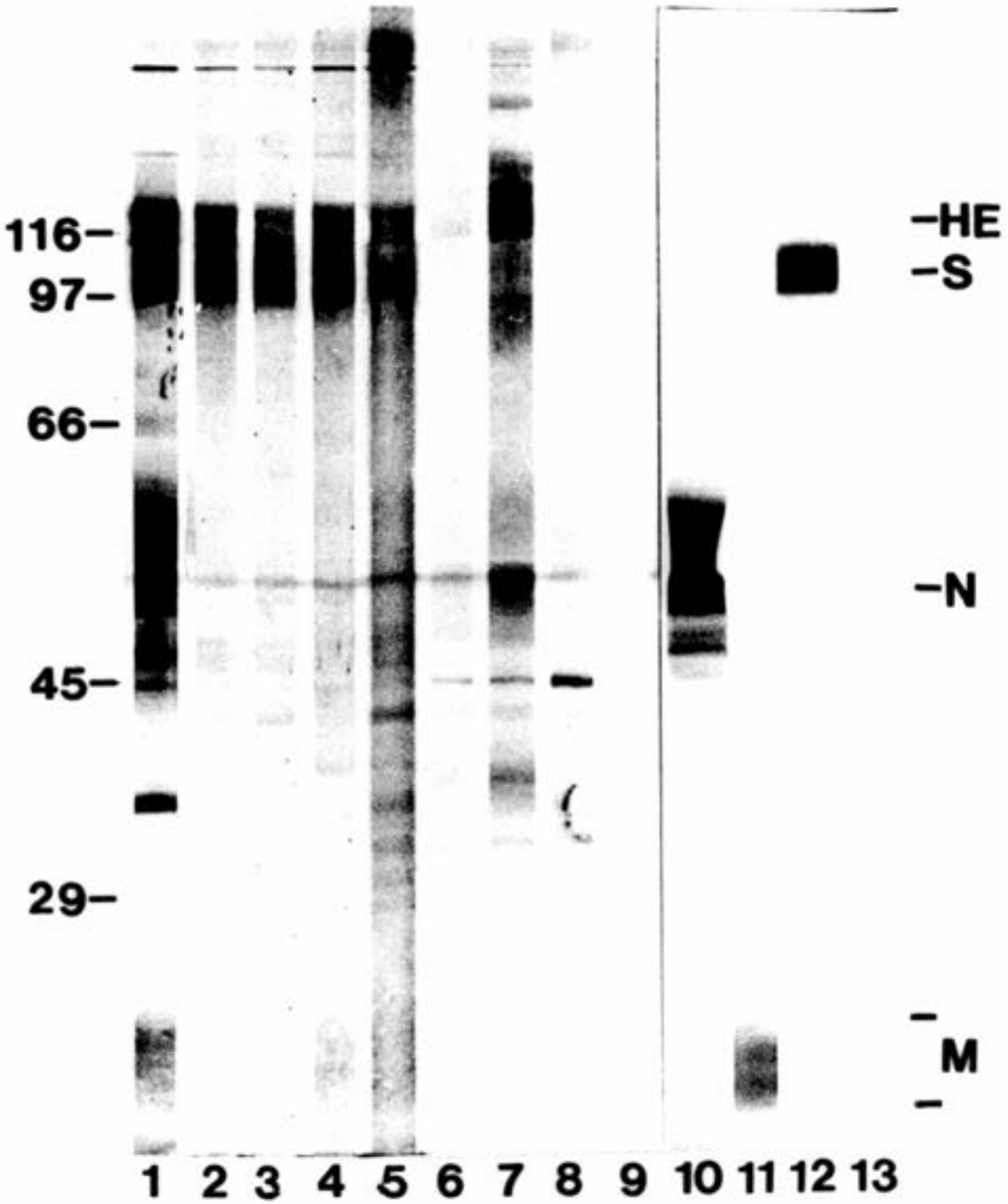
Figure 5.1

Characterisation of S2 virus proteins by Western blotting (non-reducing conditions)

The proteins of S2 strain BCV were separated by SDS/PAGE, blotted onto nitrocellulose paper and probed with polyclonal sera and MAbs. Bound Abs were detected with anti-species HRP conjugates.

Template

Lane No.	Probe
1	Gnotobiotic calf anti-S2 serum (serum 5890)
2	)
3	)
4	) Cow field serum samples
5	)
6	Gnotobiotic lamb anti-MI HRT-18 cell serum (serum 5923)
7	Gnotobiotic lamb anti-MI HRT-18 cell serum (serum 5926)
8	Gnotobiotic lamb anti-rotavirus serum (serum 3626)
9	PBS/0.5%T
10	S2 MAb: S2/8 (N)
11	CVL MAb: 5528 (M)
12	CVL MAb: 5529 (S)
13	S2 MAb: S2/7 (HE)


116–
97–
66–
45–
29–
–HE
–S
–N
–M
1 2 3 4 5 6 7 8 9 10 11 12 13

neat gnotobiotic lamb anti-MI HRT-18 cell serum (serum 5926) (Fig. 5.2). The MWs of the S, N and M proteins were as described above. The MW of the HE gp was reduced to 64 KD due to breakage of the disulphide bonds present in the original dimer molecules. Significantly fewer cellular proteins were detected in this experiment because the virus had been sucrose gradient purified prior to SDS/PAGE, although serum 5926 still bound to a cellular protein with a MW of 64 KD.

To confirm that the proteins detected were virus specific, MI HRT-18 cell proteins were also separated by SDS/PAGE, electroblotted onto nitrocellulose paper and probed with gnotobiotic calf anti-S2 serum (serum 5890) (Fig. 5.3) and the MAbs (data not shown). No bands were detected apart from that obtained due to non-specific binding of the HRP conjugate, demonstrating that the proteins described above are virus specific.

Gnotobiotic lamb anti-MI HRT-18 cell sera

To further confirm that these proteins are virus specific, anti-MI HRT-18 cell sera were raised in a gnotobiotic lamb and used as negative control probes in Western blotting experiments. The lamb was inoculated by the IM route with MI HRT-18 cells emulsified with FCA (first inoculation, day 0) or FIA (second, third and fourth inoculations; days 15, 26 and 36). Serum samples were collected on days 0, 7, 14, 26, 36 and 43 and used at dilutions of 1:4 to probe MI HRT-18 cell proteins (Fig. 5.3) or S2 virus proteins (Fig. 5.4), which were both separated under non-reducing conditions.

Figure 5.2

Characterisation of S2 virus proteins by Western blotting (reducing conditions)

The proteins of sucrose gradient purified S2 strain BCV were separated by SDS/PAGE, blotted onto nitrocellulose paper and probed with gnotobiotic lamb anti-MI HRT-18 cell serum (serum 5926) (lane 1) and gnotobiotic calf anti-S2 serum (serum 5890) (lane 2). Bound Abs were detected with anti-sheep HRP conjugate.

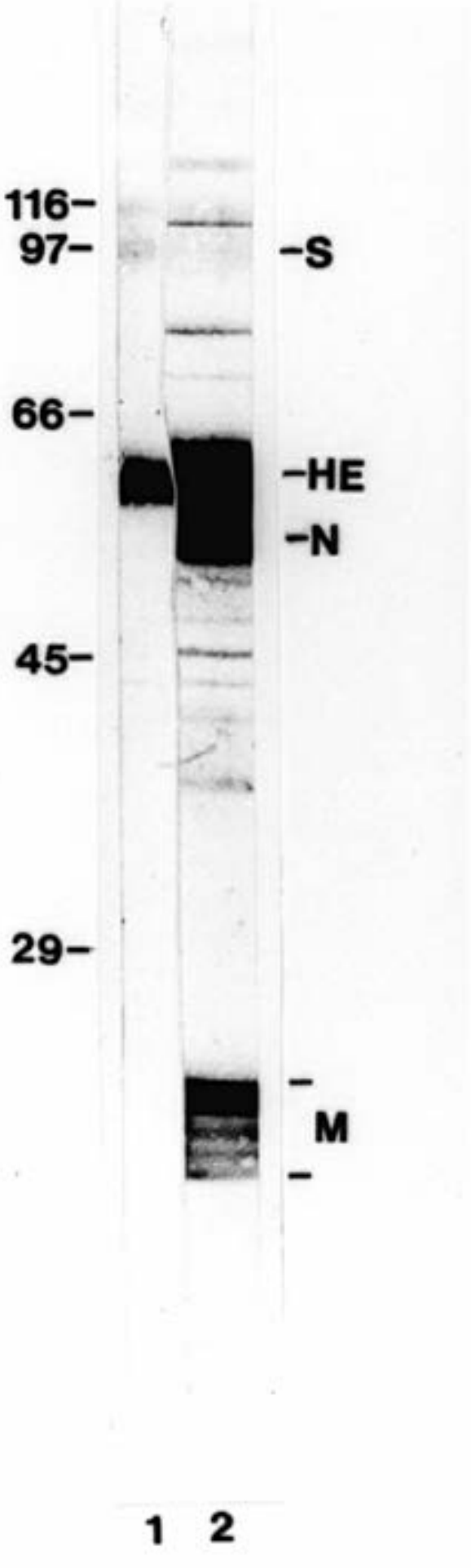

116–
97–
66–
45–
29–
–S
–HE
–N
–
M
–
1 2

Figure 5.3

MI HRT-18 cell proteins probed with gnotobiotic lamb anti-MI HRT-18 cell sera
(non-reducing conditions)

MI HRT-18 cell proteins were separated by SDS/PAGE, blotted onto nitrocellulose paper and probed with polyclonal sera. Bound Abs were detected with anti-sheep HRP conjugate.

Template

Lane No.	Probe
1	Lamb samples (0
2	(7
3	days after inoculation: (14
4	(26
5	(36 (serum 5923)
6	(43 (serum 5926)
7	Controls: Gnotobiotic calf anti-S2 serum (serum 5919)
8	Gnotobiotic calf anti-S2 serum (serum 5890)
9	Gnotobiotic lamb anti-rotavirus serum (serum 3626)

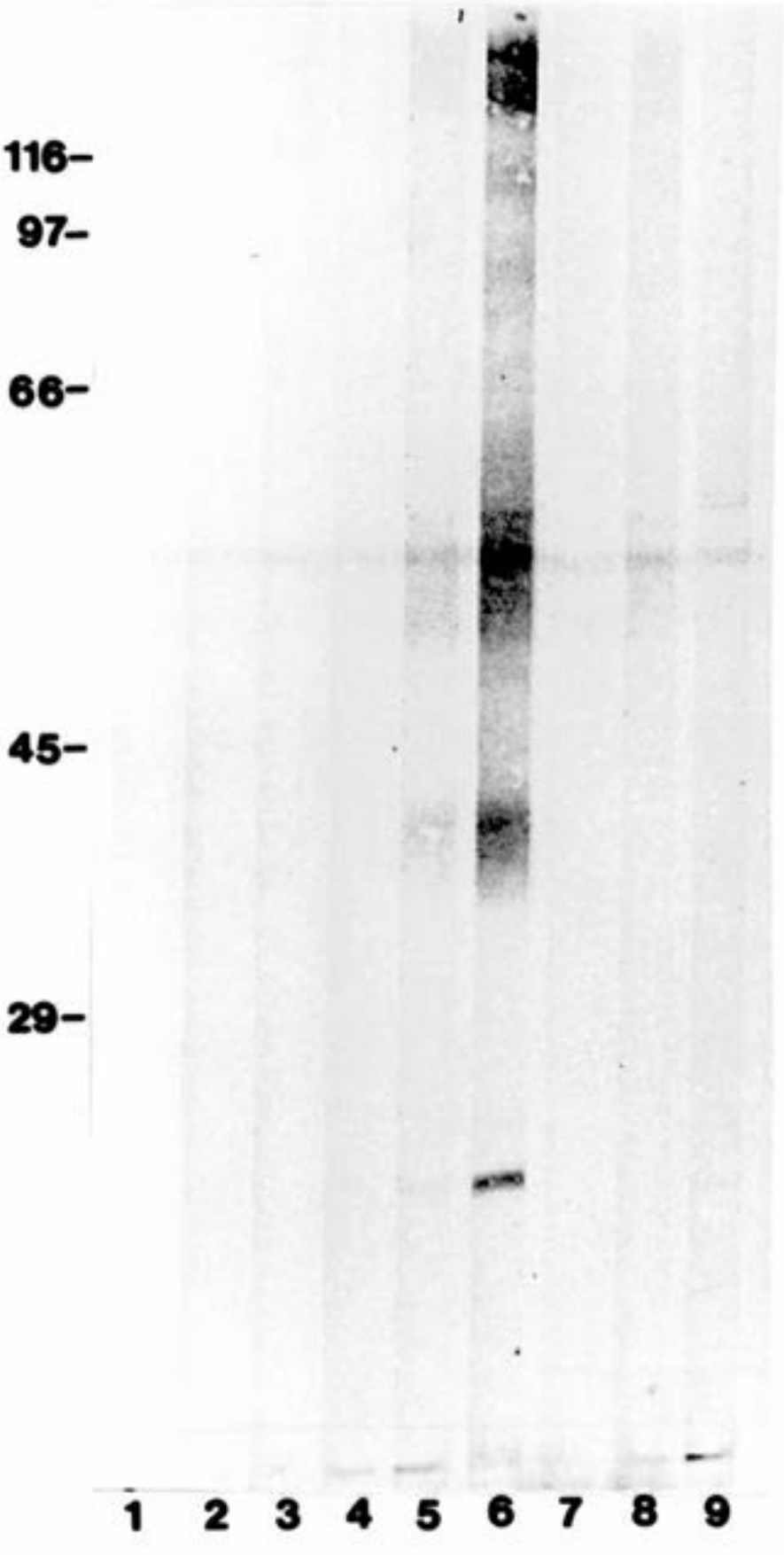

116–
97–
66–
45–
29–
1
2
3
4
5
6
7
8
9

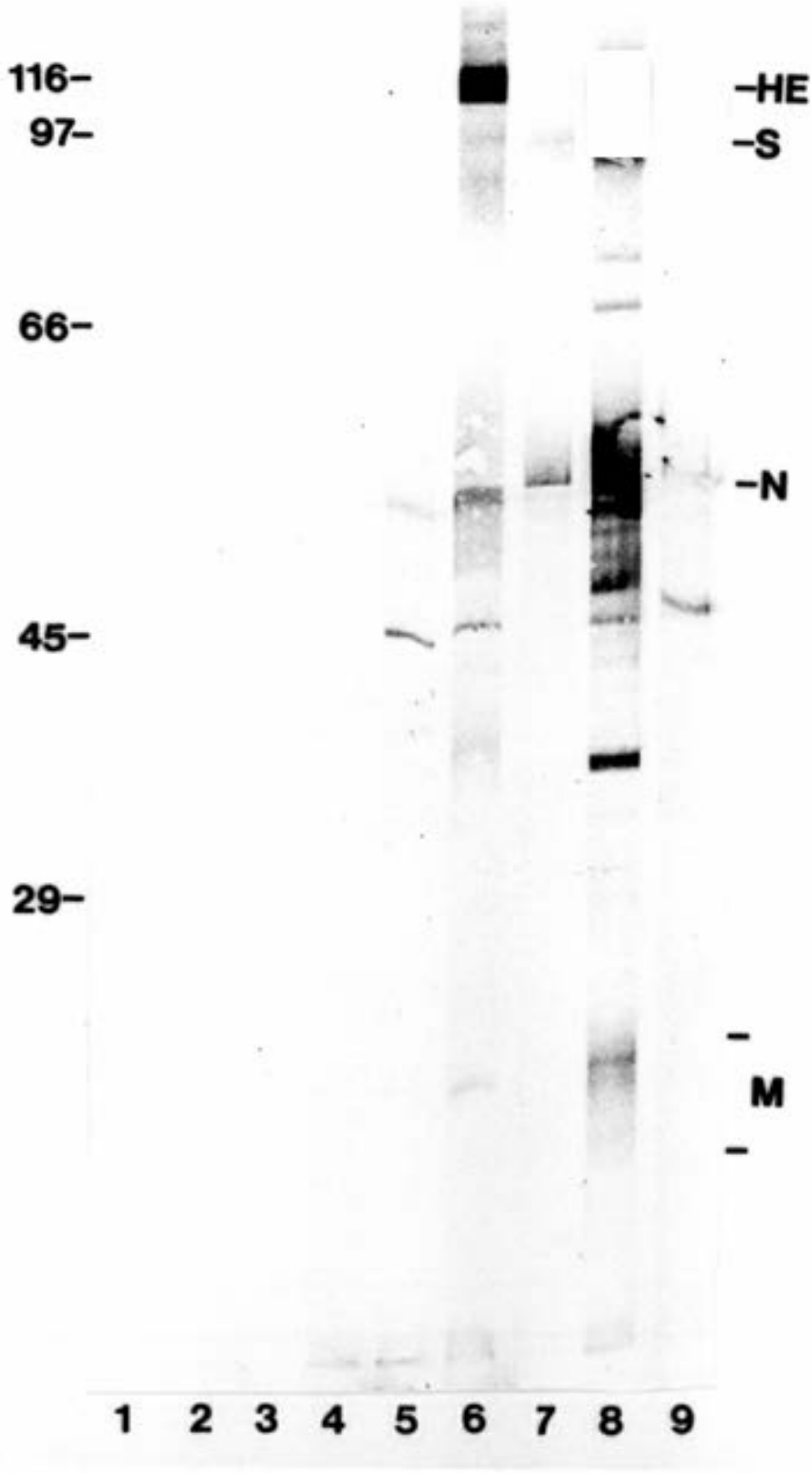
Figure 5.4

S2 virus proteins probed with gnotobiotic lamb anti-MI HRT-18 cell sera (non-reducing conditions)

The proteins of S2 strain BCV were separated by SDS/PAGE, blotted onto nitrocelluose paper and probed with polyclonal sera. Bound Abs were detected with anti-sheep HRP conjugate.

Template

Lane No.	Probe
1	Lamb samples (0
2	(7
3	days after inoculation: (14
4	(26
5	(36 (serum 5923)
6	(43 (serum 5926)
7	Controls: Gnotobiotic calf anti-S2 serum (serum 5919)
8	Gnotobiotic calf anti-S2 serum (serum 5890)
9	Gnotobiotic lamb anti-rotavirus serum (serum 3626)


116–
97–
66–
45–
29–
–HE
–S
–N
–
M
–
1 2 3 4 5 6 7 8 9

HRT-18 cell specific Abs were first detected clearly by probing blotted proteins with serum collected on day 36 (serum 5923). The serum collected on day 43 (serum 5926) stained these proteins much more strongly. The most obvious bands had MWs of 116, 102, 94, 51-->61, 38 and 22 KD (Figs. 5.3 and 5.4). The 102, 51-->61 and 22 KD bands lay in the regions corresponding to the S, N and M BCV proteins respectively, but they were unlikely to be confused with viral proteins as the cellular bands were narrower and stained less strongly (Figs. 5.1 and 5.4). The 116 KD MW cell protein stained strongly with anti-MI HRT-18 cell serum, and could potentially be confused with the HE gp (Figs. 5.1 and 5.4). Under reducing conditions anti-MI HRT-18 cell serum detected a protein with a similar MW to that of the HE gp monomer (Fig. 5.2). These results emphasised the importance of using adequate controls in Western blotting experiments.

Radiolabelling/Radioimmunoprecipitation

Attempts to determine the MWs of viral proteins by RIP were less successful. Growth curves demonstrated that release of infectious virus particles from HRT-18 cells commenced at 6-9 h pi(Figs. 3.1 and 3.2). In radiolabelling experiments ^{35}S-methionine was therefore added 5 h pi.

Radiolabelling time course

A radiolabelling time course experiment was conducted to determine the optimal duration of radiolabelling viral proteins. The radiolabel, ^{35}S-methionine, was added 5 h pi and cell lysates

from MI and S2 virus infected cells harvested 0, 1, 3, 6, 9, 21, 28, 45 and 49 h later. Scintillation counts revealed that the uptake of radiolabel increased with the duration of radiolabelling but there were no significant differences between MI and S2 infected cells (data not shown). The harvests were run on protein gels, and though autoradiography revealed many bands, none were virus specific (Fig. 5.5).

Solubilised cell lysates were immunoprecipitated with rabbit anti-S2 serum (serum 5317) and protein A sepharose and run on protein gels. Autoradiography revealed a series of proteins which had been precipitated from both MI and virus infected cells. Precipitation of cellular proteins was expected because serum 5317 had been raised against unpurified S2 virus. A virus specific doublet of bands with MWs of 50 and 52 KD was detected (N protein). These bands were first observed 6 h after adding the radiolabel and increased in intensity until around 28 h (Fig. 5.6). Subsequent experiments employed a 26 h period of radiolabelling.

Titration of unlabelled methionine

Better radiolabelling of the virus proteins was required. In an attempt to increase cellular uptake of ^{35}S- methionine, unlabelled methionine was included at concentrations of 0, 5, 10, 15, 20 and 50% relative to the levels normally present in media. The supernates and cell lysates were harvested 26 h after adding the radiolabel. Scintillation counts demonstrated that uptake of radiolabel was roughly constant when unlabelled methionine was included at levels of 0 to 15%, but dropped rapidly beyond these

Figure 5.5

Radiolabelling time course: Protein gel (reducing conditions)

^{35}S-methionine was added to MI and S2 virus infected HRT-18 cells at 5 h pi. The cell lysates were harvested after appropriate incubation periods and run on a protein gel. Radiolabelled proteins were detected by autoradiography: the autorad was developed after 7 days.

Template

Lane		Contents	Duration of radio-labelling/h
1	Cell lysates:	MI	0
2		S2	
3		MI	1
4		S2	
5		MI	3
6		S2	
7		MI	6
8		S2	
9		MI	9
10		S2	
11		MI	21
12		S2	
13		MI	28
14		S2	
15		MI	45
16		S2	
17		MI	49
18		S2	

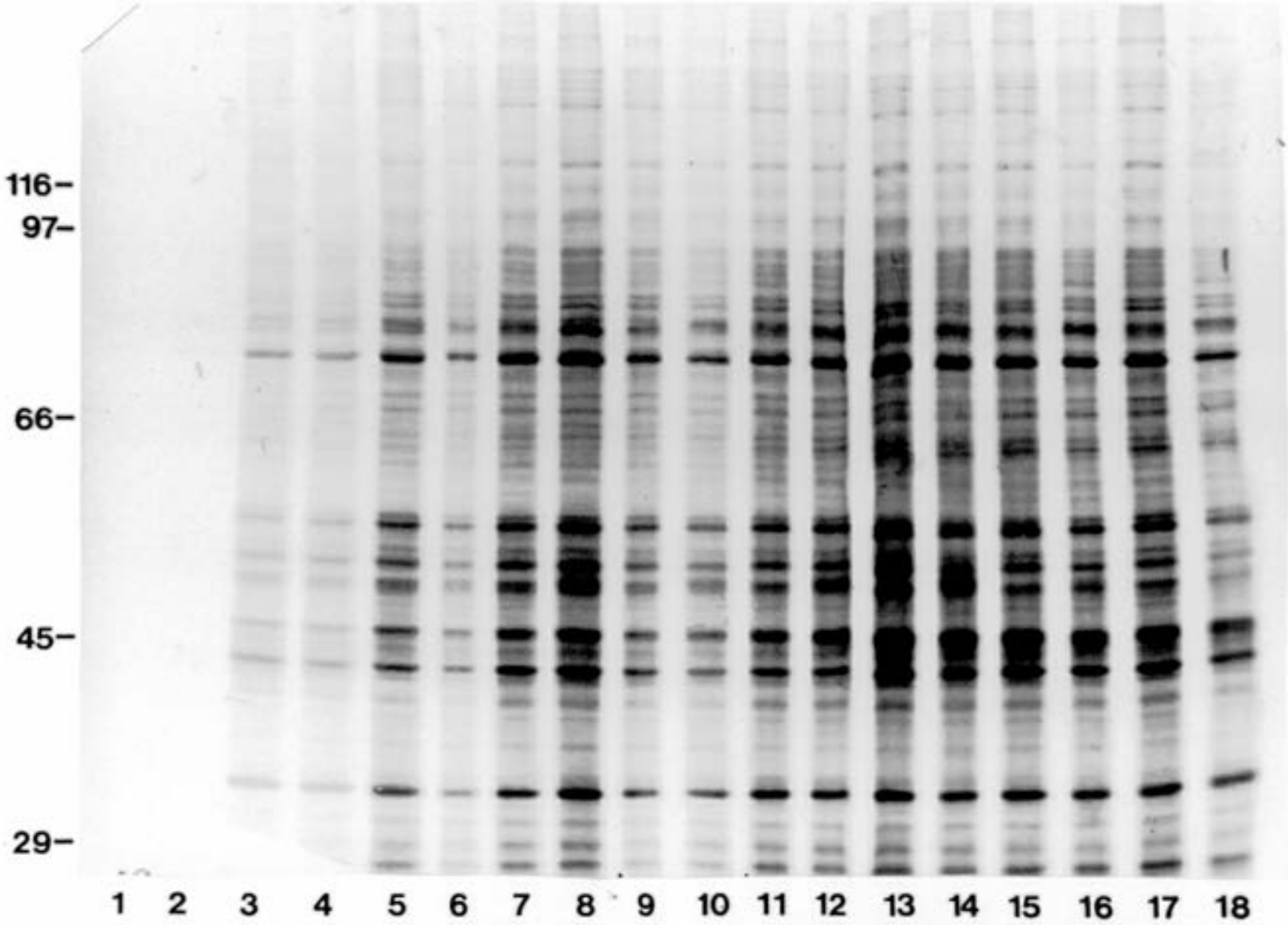

116–
97–
66–
45–
29–
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

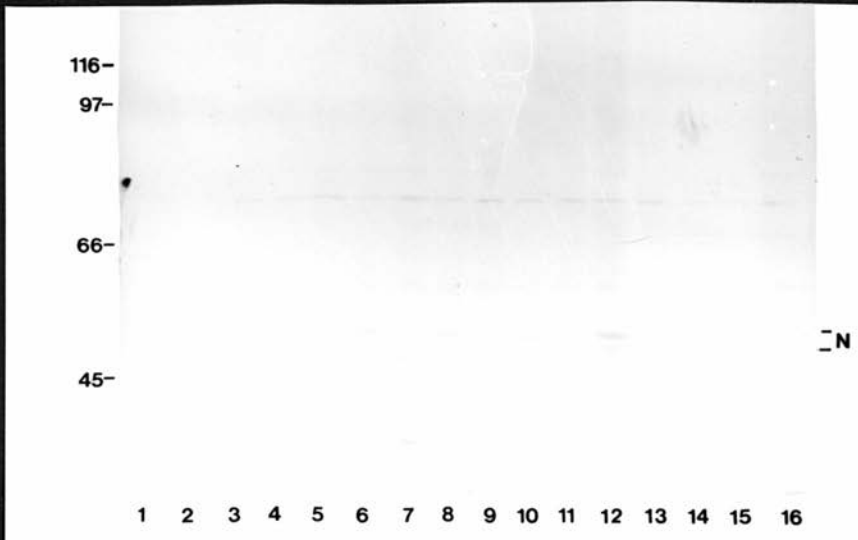
Figure 5.6

Radiolabelling time course: RIP (reducing conditions)

^{35}S-methionine was added to MI and S2 virus infected HRT-18 cells at 5 h pi. The cell lysates were harvested after appropriate incubation periods and immunoprecipitated with rabbit anti-S2 serum (serum 5317). Precipitated proteins were separated by SDS/PAGE and radiolabelled proteins detected by autoradiography: the autorad was developed at 14 days.

Template

Lane	Contents	Duration of radiolabelling/h
Cell lysates:		
1	MI	1
2	S2	
3	MI	3
4	S2	
5	MI	6
6	S2	
7	MI	9
8	S2	
9	MI	21
10	S2	
11	MI	28
12	S2	
13	MI	45
14	S2	
15	MI	49
16	S2	


116–
97–
66–
45–
N
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

levels. There were no significant differences between S2 infected and MI cells (data not shown). Autoradiography revealed many bands when the radiolabelled harvests were run on protein gels, including a single virus specific band with a MW of 53 KD (N protein) which was detected in the supernates only. This band was very intense when unlabelled methionine was included at levels of 0 to 15%, but was less obvious in the presence of higher concentrations of methionine (Fig. 5.7).

The cell lysates and supernates were immunoprecipitated with serum 5317 and protein A sepharose and run on protein gels. Autoradiography revealed a doublet of virus specific bands with MWs of 50 + 52 KD (N protein). The concentration of unlabelled methionine had little effect on the precipitation of this protein but the signal was stronger from supernates than cell lysates (Fig. 5.8).

Epitope mapping

MAb purification and biotinylation

The MAbs directed against S2 strain BCV were purified from their ascitic fluids by affinity chromatography on a protein A sepharose column. This procedure was performed by Dr. R. Davies. The MAbs all eluted off the column at the pHs predicted from their isotypes (Table 4.1). Small peaks were also observed which represented the elution of the other Igs present in mouse ascitic fluids. Purified MAbs were titrated in IF, SN and HAI tests (Table 4.6). Successful purification had been achieved as the MAbs retained their original activities in these tests.

Figure 5.7

Titration of unlabelled methionine: Protein gel (reducing conditions)

^{35}S-methionine was added to MI and S2 virus infected HRT-18 cells at 5 h pi. Unlabelled methionine was included in the incubation media at various concentrations. The supernates were harvested 26 h after adding the radiolabel and run on a protein gel. Radiolabelled proteins were detected by autoradiography: the autorad was developed after 14 days.

Template

Lane	Contents		Methionine: % of normal level
1	supernates:	MI)	0
2		S2)	
3		MI)	5
4		S2)	
5		MI)	10
6		S2)	
7		MI)	15
8		S2)	
9		MI)	20
10		S2)	
11		MI)	50
12		S2)	

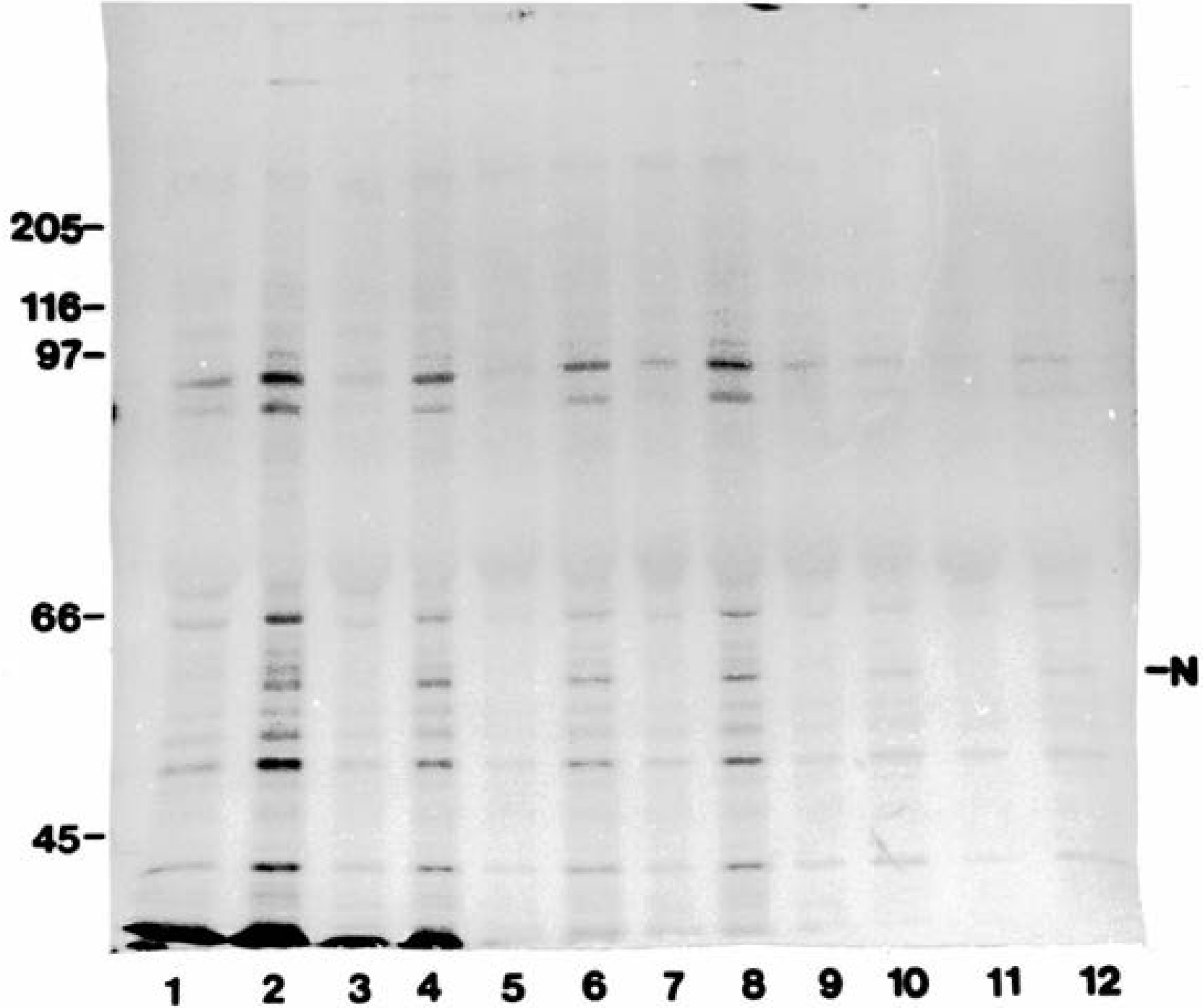

205-
116-
97-
66-
45-
-N
1
2
3
4
5
6
7
8
9
10
11
12

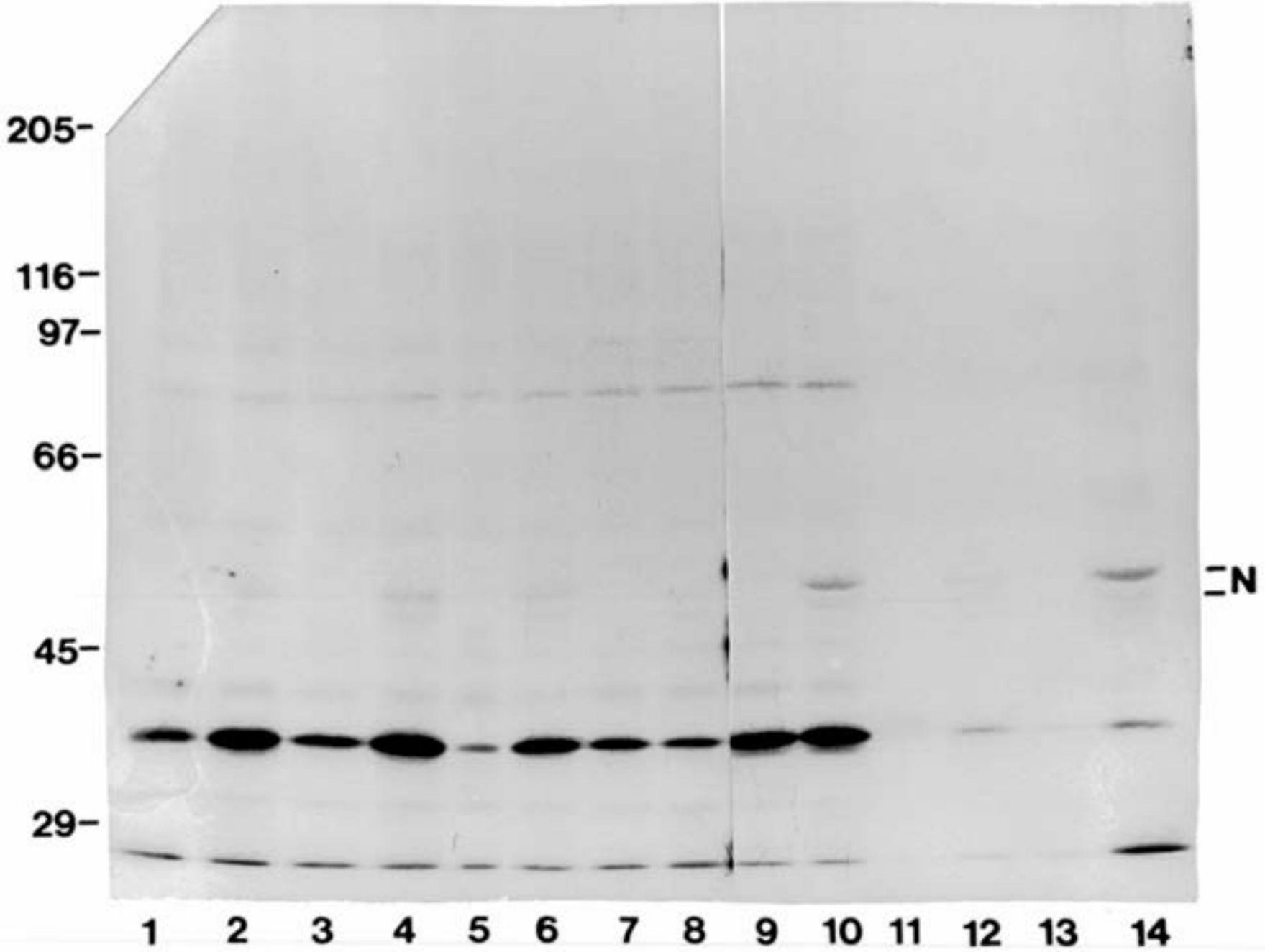
Figure 5.8

Titration of unlabelled methionine: RIP (reducing conditions)

^{35}S-methionine was added to MI and S2 virus infected HRT-18 cells at 5 h pi. Unlabelled methionine was included in the incubation media at various concentrations. The cell lysates and supernates were harvested 26 h after adding the radiolabel and immunoprecipitated with rabbit anti-S2 sera (serum 5317). Precipitated proteins were separated by SDS/PAGE and radiolabelled proteins detected by autoradiography: the autorad was developed at 40 days.

Template

Lane		Contents		Methionine: % of normal level
1	cell	MI	)	0
2	lysates:	S2	)	
3		MI	)	5
4		S2	)	
5		MI	)	10
6		S2	)	
7		MI	)	15
8		S2	)	
9		MI	)	50
10		S2	)	
11	cell	MI	)	10
12	pellets:	S2	)	
13	supernates:	MI	)	10
14		S2	)	


205-
116-
97-
66-
45-
29-
N
1
2
3
4
5
6
7
8
9
10
11
12
13
14

The purified MAbs were used in ELISAs to construct binding affinity curves and as competitor Abs in competition ELISAs. Aliquots of each MAb were biotinylated (MAb-B) and used as detecting Abs in both competition ELISAs and epitope blocking assays (EBAs).

MAb binding affinity curves

Purified MAbs were titrated in ELISAs using anti-mouse HRP conjugate as the detecting Ab. Binding affinity curves were constructed by plotting the OD readings against log ug MAb/well (Figs. 5.9 and 5.10). The OD values reached at the plateaux of these curves are an approximate measure of binding affinity (Deregt and Babiuk, 1987). The curves obtained with the 4 HE MAbs were almost identical, demonstrating that these MAbs all have similar binding affinities. The 3 N MAbs and the single S MAb all gave lower OD readings than the HE MAbs. Two of the 3 N MAbs failed to reach saturation levels at the concentrations tested. Non-specific binding of the MAbs to control wells coated with MI HRT-18 cell harvests was negligible (OD readings $\leq$0.12 at 5 ug Ig/well).

Competition ELISAs

The optimal conditions for performing competition ELISAs were first determined. Chequerboard titrations were performed to determine the maximal dilutions of coating Ab (lamb anti-BCV serum, serum 5000) and S2 virus which gave OD readings of about 1.0 in the absence of competitor Abs. Serum 5000 was used at a dilution of 1:6400 in all the competition assays and S2 virus was used neat

Figure 5.9

Binding affinity curves

MAbs directed against the HE and S gps

Binding affinity curves

MAbs directed against the HE and S gps

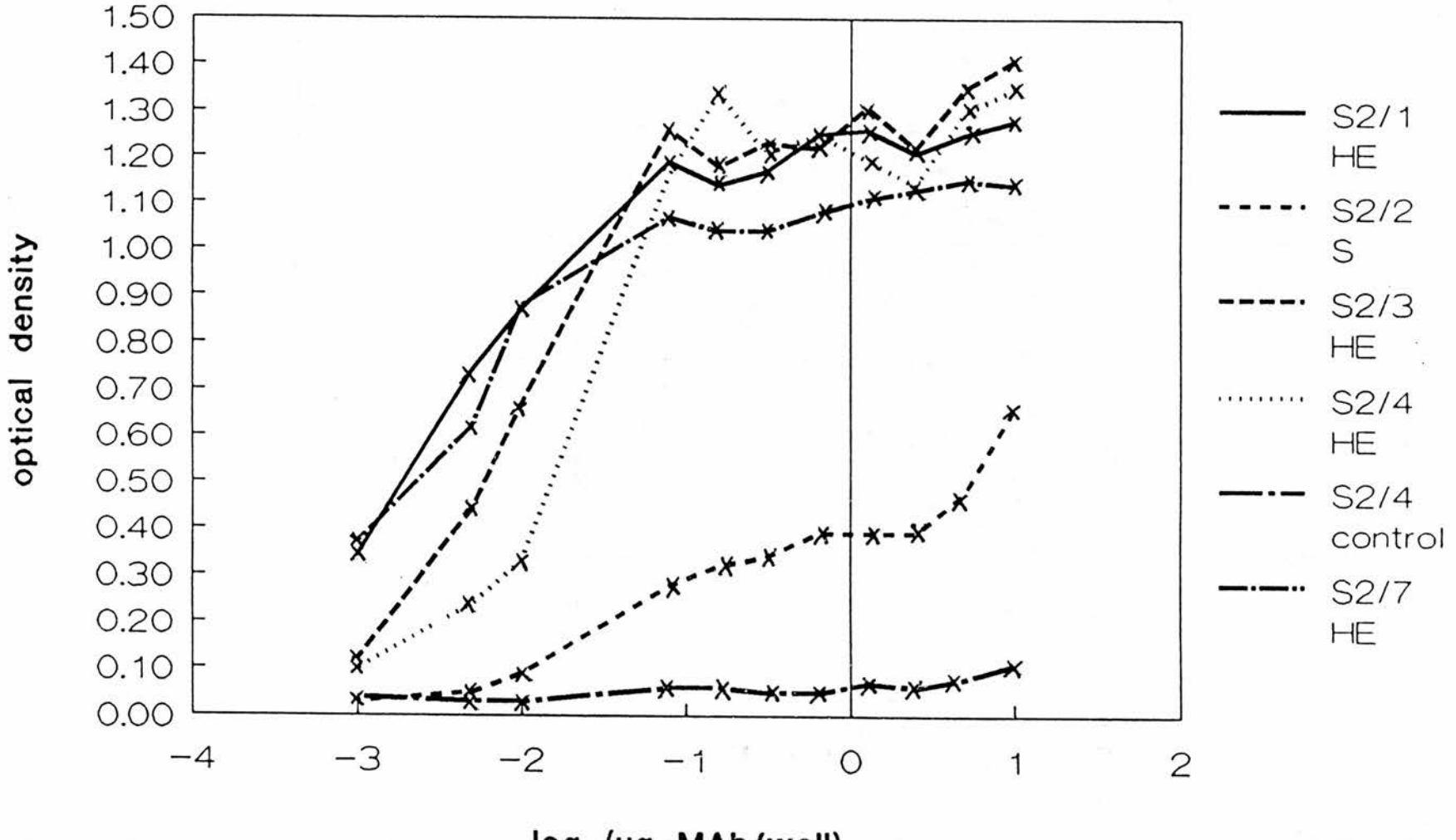
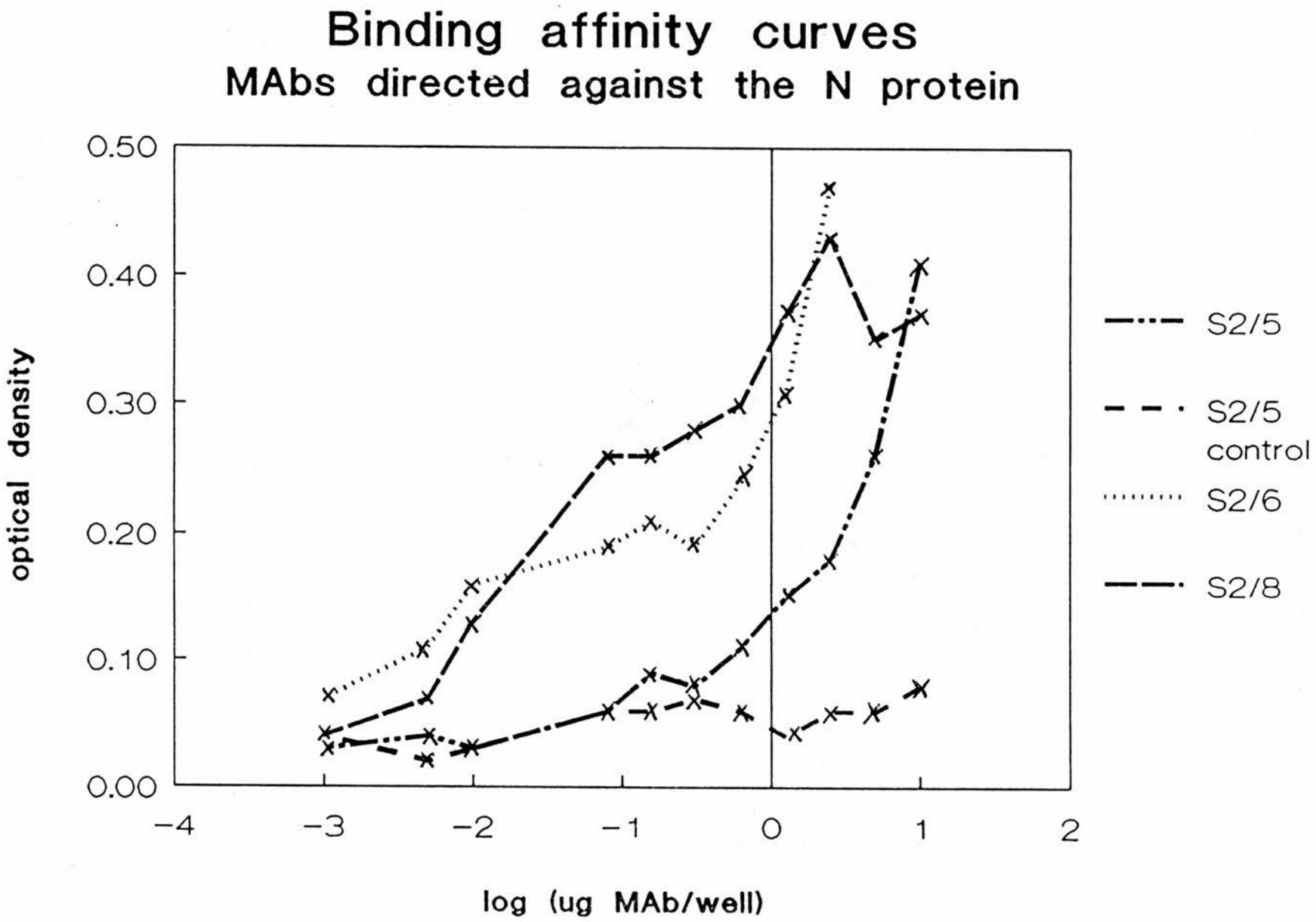


Figure 5.10

Binding affinity curves

MAbs directed against the N protein


Binding affinity curves
MAbs directed against the N protein
optical density
0.50
0.40
0.30
0.20
0.10
0.00
−4
−3
−2
−1
0
1
2
log (ug MAb/well)
S2/5
S2/5 control
S2/6
S2/8

with biotinylated N MAbs and at dilutions of 1:4 with biotinylated HE and S MAbs. Chequerboard titrations were also performed with the biotinylated MAbs and avidin/HRP conjugate and appropriate dilutions chosen to give approximately 90% saturation of the virus (Table 5.1).

The biotinylated MAbs were used as detecting Abs in competition ELISAs. MAbs directed against the same proteins were tested in 2-way competition tests whilst the other MAbs were used as negative control competitor Abs. ODs were measured at standard concentrations (ug/ml) of competitor MAb/well, and % competition values calculated by comparison with the OD readings obtained in the absence of competitor Abs. Binding of biotinylated HE and S MAbs to MI HRT-18 cells was found to be negligible (OD values $\leq$0.04) and was therefore ignored in these calculations. The N MAbs gave higher levels of non-specific binding to MI HRT-18 cells (OD values $\leq$0.10), so the average OD values obtained from these wells were subtracted from the OD values used in the calculations. Competition curves were constructed by plotting the % competition values against ug competitor MAb/well. The % competition at the plateaux of these curves was defined as strong ++ (>70%), moderate + (<70% and >20%) or weak - (< 20%).

Each MAb was tested for its ability to compete with itself by using the same MAb as competitor and detecting Abs. High levels of self competition were achieved with all the HE and S MAbs (>85% at the plateau level) and rather lower levels with the N MAbs (69% at 1 ug/well for MAb S2/6 and 54% at 2 ug/well for MAb S2/8) (Figs. 5.11 to 5.17).

Table 5.1

Competition ELISAs

Dilutions of detecting MAbs and avidin / HRP conjugate

Protein specificity	Detecting MAb	Dilution	
		Detecting MAb	Avidin / HRP
HE	S2/1	1:8000	1:8000
	S2/3	1:2000	1:8000
	S2/4	1:2000	1:8000
	S2/7	1:4000	1:8000
S	S2/2	1:800	1:2000
N	S2/5*	-	-
	S2/6	1:400	1:2000
	S2/8	1:800	1:2000

* MAb S2/5 was not used as a biotinylated detecting Ab.

Figure 5.11

Competition ELISAs

Results obtained using MAb S2/1 as the detecting Ab

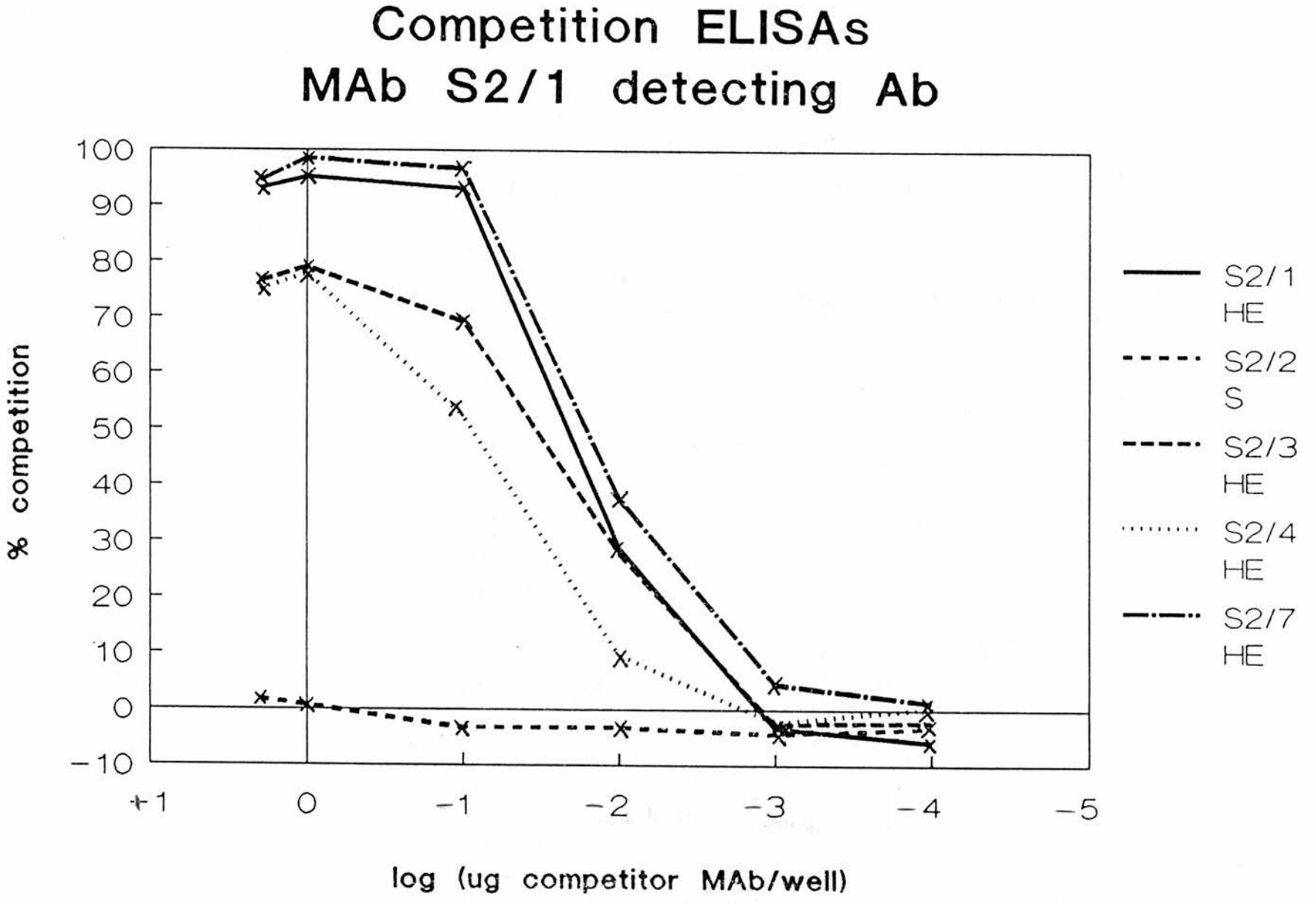

Competition ELISAs
MAb S2/1 detecting Ab
% competition
100
90
80
70
60
50
40
30
20
10
0
−10
+1
0
−1
−2
−3
−4
−5
log (ug competitor MAb/well)
S2/1 HE
S2/2 S
S2/3 HE
S2/4 HE
S2/7 HE

Figure 5.12

Competition ELISAs

Results obtained using MAb S2/3 as the detecting Ab

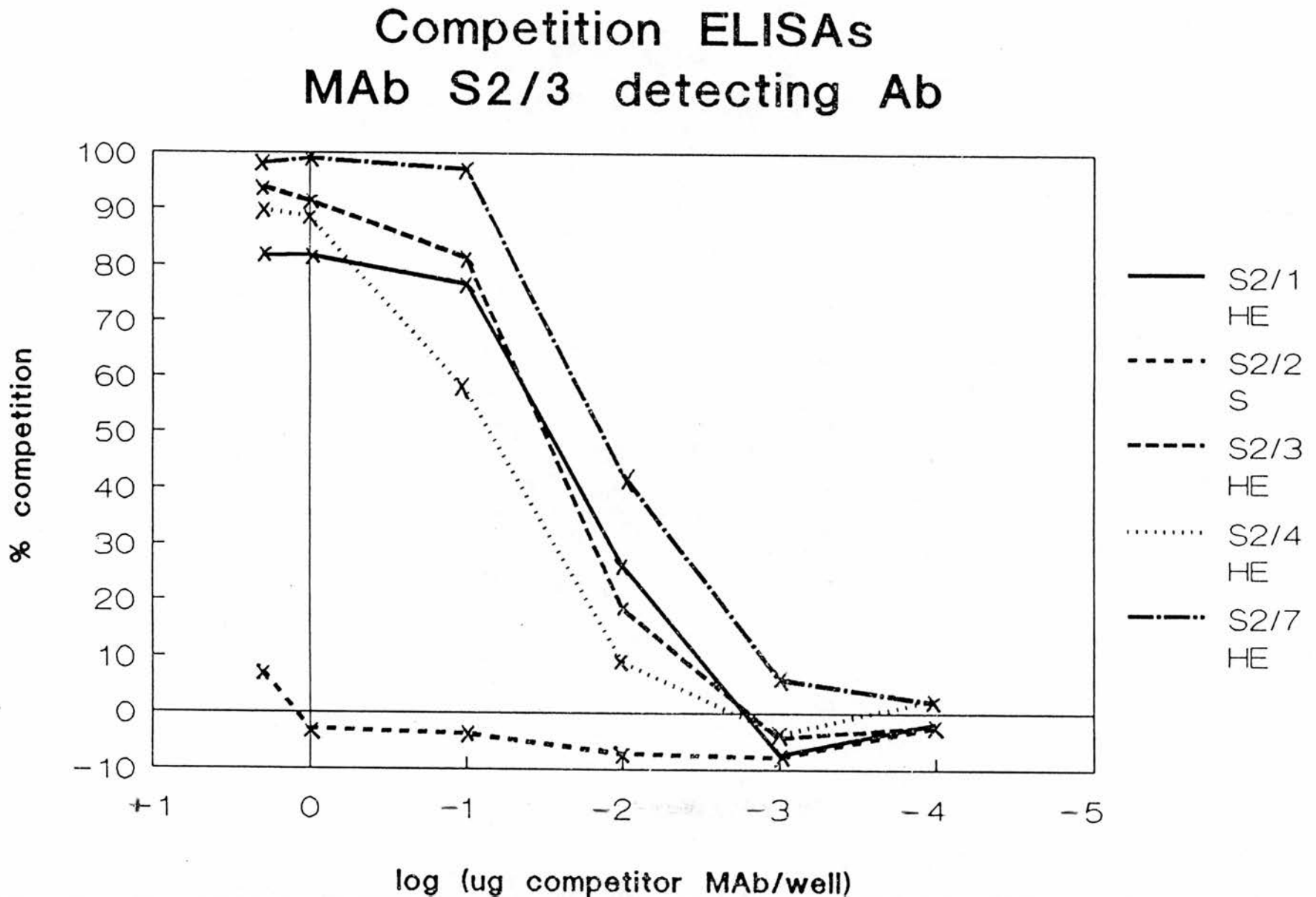

Competition ELISAs
MAb S2/3 detecting Ab
% competition
100
90
80
70
60
50
40
30
20
10
0
-10
+1
0
-1
-2
-3
-4
-5
log (ug competitor MAb/well)
S2/1 HE
S2/2 S
S2/3 HE
S2/4 HE
S2/7 HE

Figure 5.13

Competition ELISAs

Results obtained using MAb S2/4 as the detecting Ab

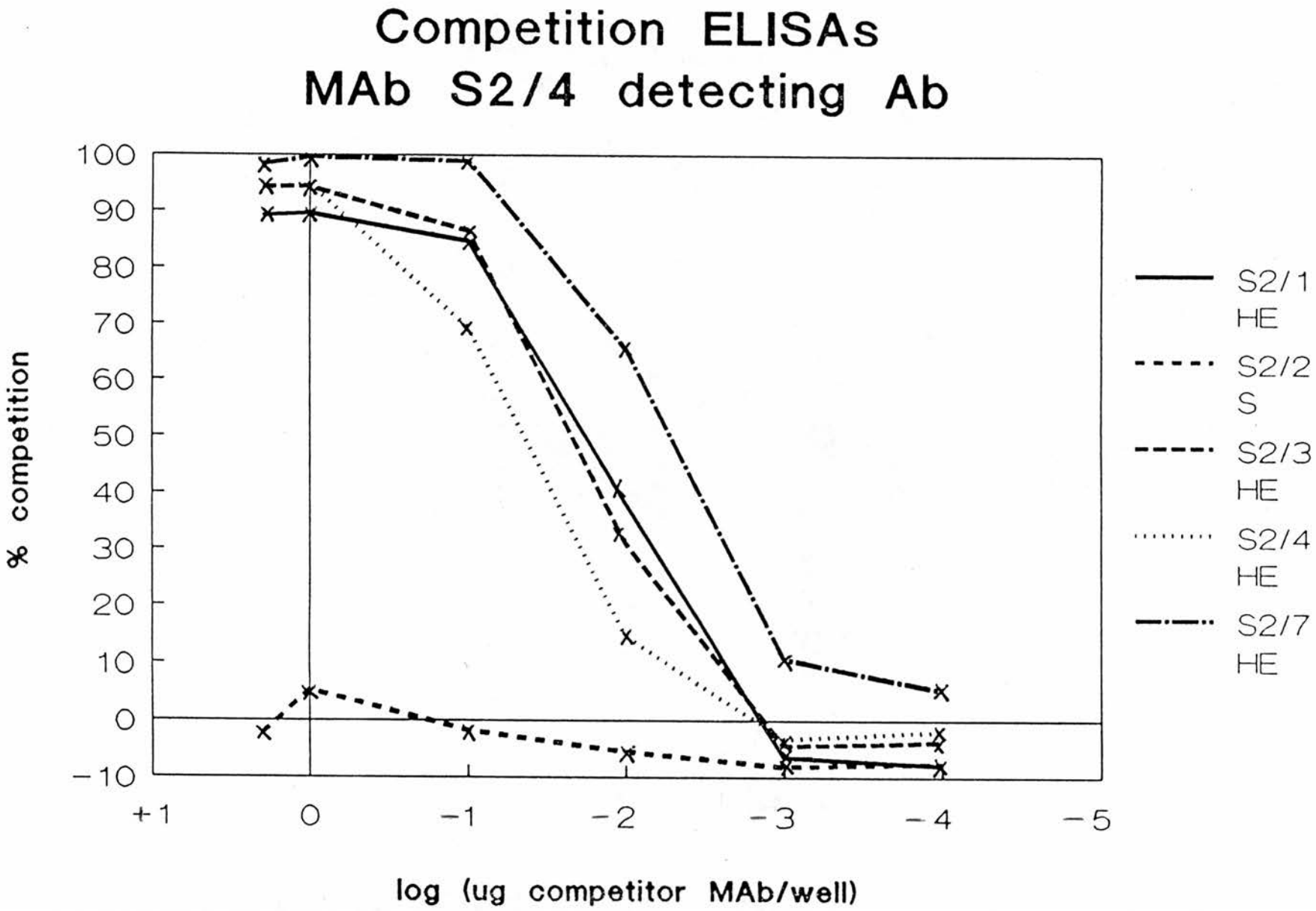

Competition ELISAs
MAb S2/4 detecting Ab
% competition
100
90
80
70
60
50
40
30
20
10
0
−10
+1
0
−1
−2
−3
−4
−5
log (ug competitor MAb/well)
S2/1 HE
S2/2 S
S2/3 HE
S2/4 HE
S2/7 HE

Figure 5.14

Competition ELISAs

Results obtained using MAb S2/7 as the detecting Ab

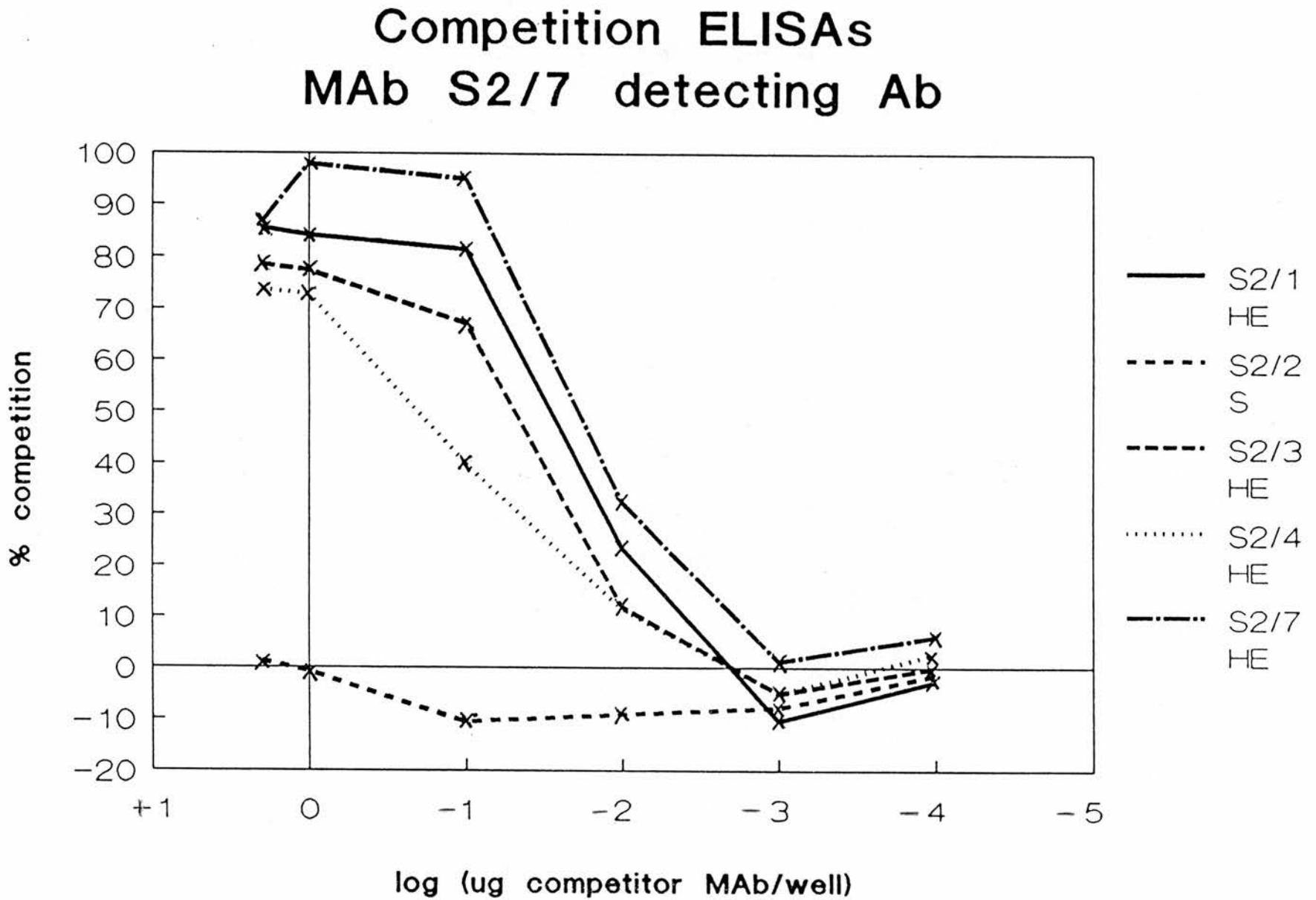

Competition ELISAs
MAb S2/7 detecting Ab
% competition
100
90
80
70
60
50
40
30
20
10
0
−10
−20
+1
0
−1
−2
−3
−4
−5
log (ug competitor MAb/well)
S2/1 HE
S2/2 S
S2/3 HE
S2/4 HE
S2/7 HE

Figure 5.15

Competition ELISAs

Results obtained using MAb S2/2 as the detecting Ab

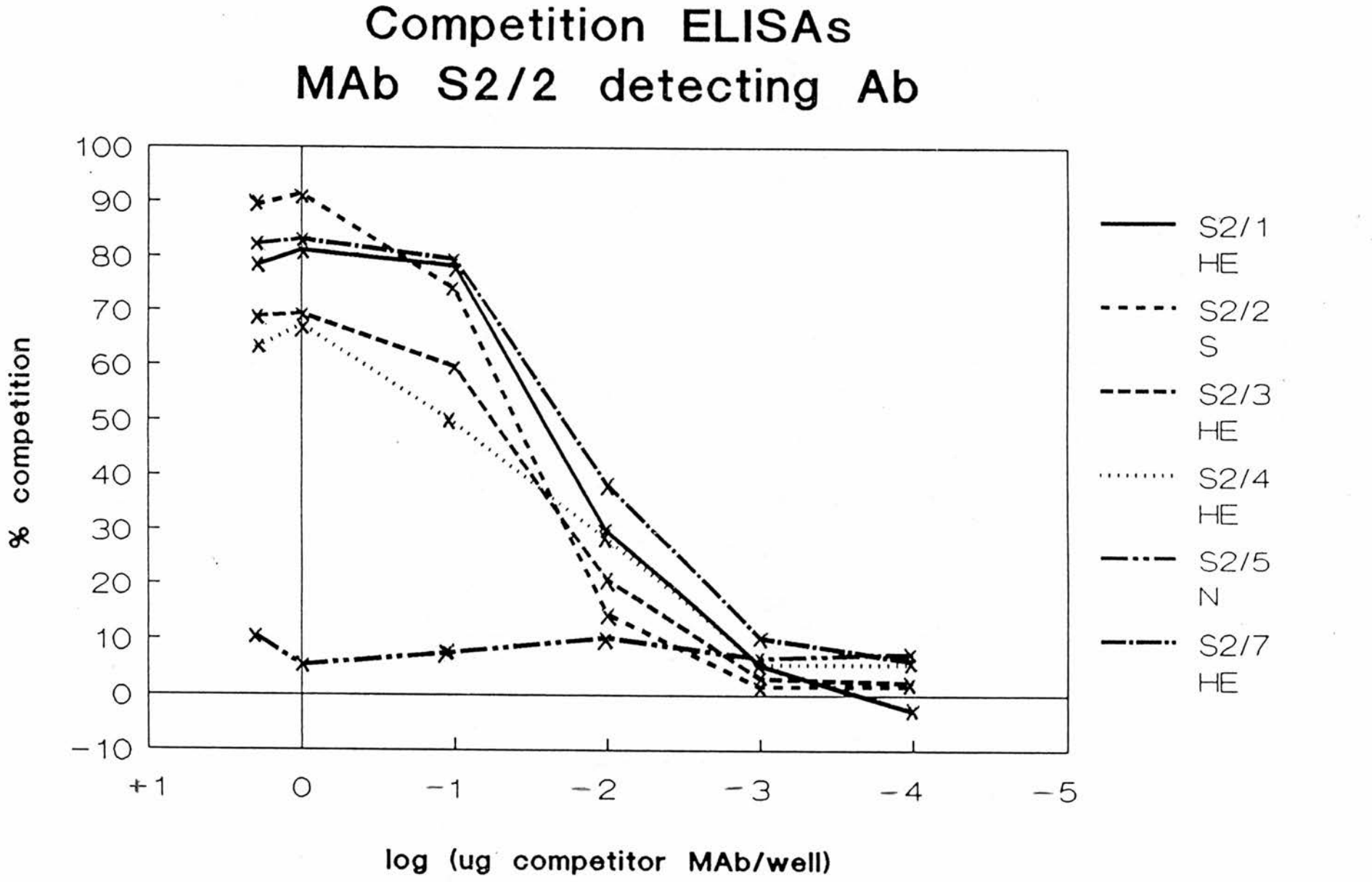

Competition ELISAs
MAb S2/2 detecting Ab
% competition
100
90
80
70
60
50
40
30
20
10
0
−10
+1
0
−1
−2
−3
−4
−5
log (ug competitor MAb/well)
S2/1 HE
S2/2 S
S2/3 HE
S2/4 HE
S2/5 N
S2/7 HE

Figure 5.16

Competition ELISAs

Results obtained using MAb S2/6 as the detecting Ab

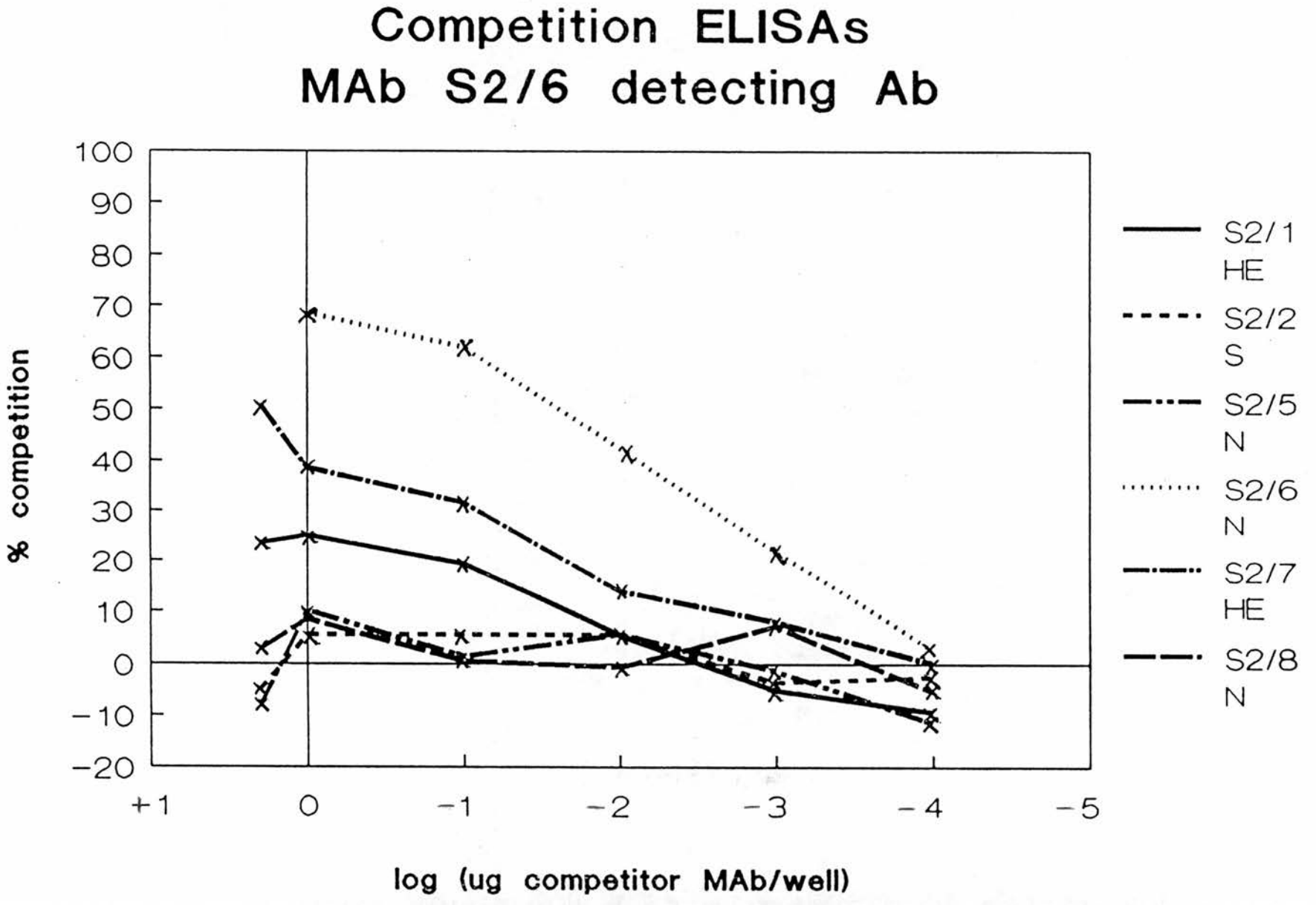
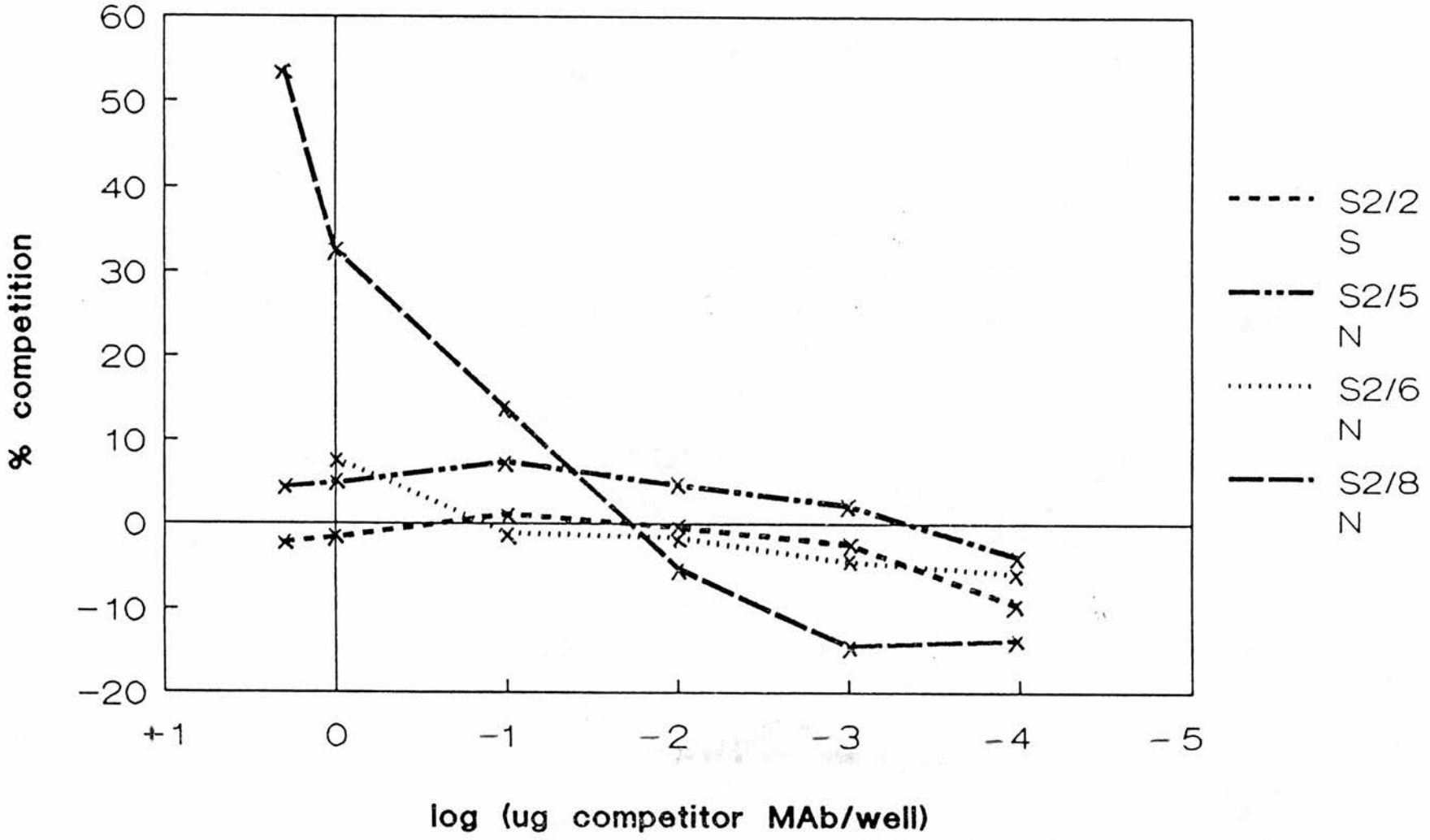

Competition ELISAs
MAb S2/6 detecting Ab
% competition
100
90
80
70
60
50
40
30
20
10
0
−10
−20
+1
0
−1
−2
−3
−4
−5
log (ug competitor MAb/well)
S2/1 HE
S2/2 S
S2/5 N
S2/6 N
S2/7 HE
S2/8 N

Figure 5.17

Competition ELISAs

Results obtained using MAb S2/8 as the detecting Ab

Competition ELISAs
MAb S2/8 detecting Ab



HE and S gps

The results obtained using the HE and S MAbs as biotinylated detecting Abs in competition ELISAs are shown in Figs. 5.11 to 5.15. The curves obtained using N MAbs as competitor Abs have been omitted for the sake of clarity as competition with these Abs was negligible (<10% at 2 ug competitor MAb/well). Strong 2-way competition occurred between all the HE MAbs, which therefore mapped to a single antigenic region (Table 5.2). The HE MAbs also showed strong to moderate levels of competition with biotinylated S MAb (S2/2), but competition did not occur in the reverse direction.

N protein

The results obtained using the N MAbs S2/6 and S2/8 as biotinylated detecting Abs are shown in Figs. 5.16 and 5.17 and summarised in Table 5.3. The 3 N MAbs failed to compete with each other (<5% competition at 2 ug competitor MAb/well), so they mapped to 3 distinct antigenic regions. As expected, the S MAb (S2/2) failed to compete with these MAbs (<1% competition at 2 ug competitor MAb/well) as did the HE MAbs with MAb S2/8 (<1% competition at 2 ug competitor MAb/well). An unexpected finding was that the 4 HE MAbs showed moderate levels of competition with MAb S2/6 (22 to 51% competition at 2 ug competitor MAb/well) - data shown for S2/1 and S2/7 in Fig. 5.16.

It proved impossible to use MAb S2/5 as a biotinylated detecting Ab in competition ELISAs, as it bound strongly to MI HRT-18 cell harvests and failed to show self competition. Binding affinity curves were constructed before and after biotinylation, using

Table 5.2

Summary of results obtained in competition ELISAs.
Antigenic regions defined by the HE gp MAbs

Competition ELISAs

Antigenic regions defined by the HE gp MAbs

Competitor MAb	Detecting MAb *				Antigenic region
	S2/1	S2/3	S2/4	S2/7	
S2/1	++	++	++	++	1
S2/3	++	++	++	++	
S2/4	++	++	++	++	
S2/7	++	++	++	++	

* ++ = >70% competition + = <70% and > 20% competition - = < 20% competition

Table 5.3

Summary of results obtained in competition ELISAs.
Antigenic regions defined by the N protein MAbs

Competition ELISAs

Antigenic regions defined by the N protein MAbs

Competitor MAb	Detecting MAb *			Antigenic region
	S2/5	S2/6	S2/8	
S2/5	NT	-	-	1
S2/6	NT	+	-	2
S2/8	NT	-	+	3

* ++ = >70% competition + = <70% and > 20% competition - = < 20% competition

anti-mouse HRP conjugate and avidin/HRP conjugate respectively to detect bound Abs (Fig. 5.18). Its binding affinity for both virus and MI HRT-18 cell harvests increased dramatically after biotinylation, but relatively low OD values were obtained when anti-mouse HRP conjugate was used in place of avidin/HRP to detect bound biotinylated S2/5. A control MAb (S2/8) behaved as expected throughout these tests. There are 2 possible explanations to account for the abnormal behaviour of S2/5 MAb after biotinylation. The first possibility is that the high OD values obtained using avidin/HRP conjugate were due to the presence of free biotin which had not bound to the Ab. This free biotin somehow prevented binding of anti-mouse HRP conjugate to virus bound MAb. Alternatively, biotinylation of the MAb increased the binding affinity of the MAb for both virus and MI cells, but it also prevented binding of anti-mouse HRP conjugate to the MAb.

Discussion

MWs of S2 viral proteins

Western blotting

The MWs of the S2 virus proteins determined using gnotobiotic calf anti-S2 serum (serum 5890) and cow field sera as probes in Western blotting experiments confirmed the values previously obtained using MAbs (Chapter 4). These figures were similar to those reported for other strains of BCV (King and Brian, 1982; Vautherot and Laporte, 1983; Deregt *et al*, 1987). Minor variations in reported MWs may reflect genuine strain variations between different viruses or may arise because the viruses have

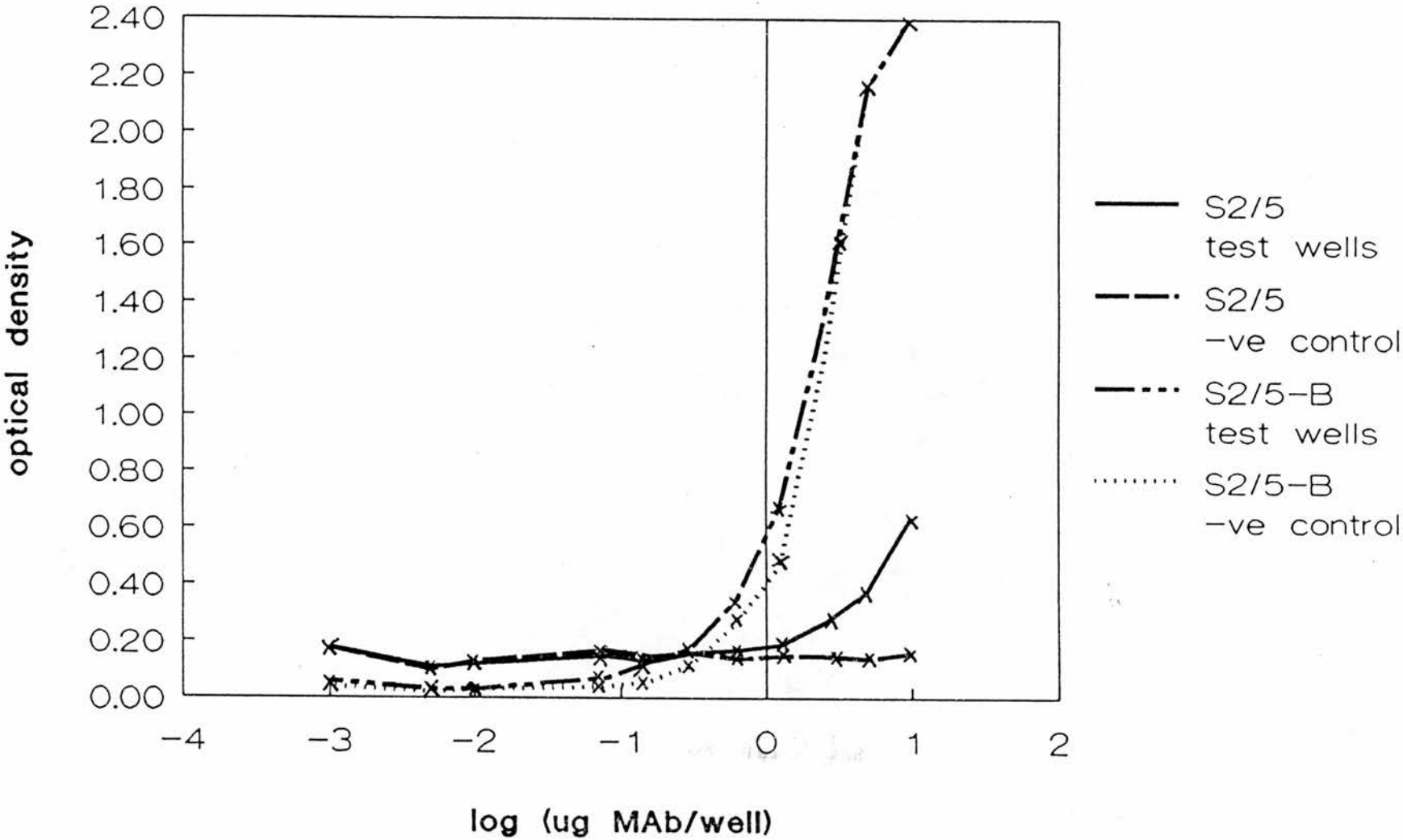
Figure 5.18

Binding affinity curves

MAb S2/5 before and after biotinylation; bound MAb detected with anti-mouse HRP conjugate and avidin/HRP conjugate respectively.

Binding affinity curves

MAb S2/5 before and after biotinylation



been grown in different cell lines. There are also often artefacts caused by the use of different experimental procedures.

In the Western blotting experiments, the N and M proteins were both seen as a series of closely migrating bands. This phenomenon has already been discussed for the N protein in Chapter 4. The M gp exists as a series of species which have different levels of glycosylation on an identical protein backbone: the M gp is therefore seen as a series of bands after SDS/PAGE (Deregt et al, 1987).

Viral specificity of the detected proteins was confirmed by using BCV specific sera and MAbs to probe blotted MI HRT-18 cell proteins, and using MI HRT-18 cell specific sera to probe blotted viral proteins. An alternative method of demonstrating that the polyclonal sera were detecting viral rather than cellular proteins would have been to absorb out Abs directed against cellular proteins prior to using these sera as probes.

Radiolabelling/Radioimmunoprecipitation

Attempts to investigate the MWs of the viral proteins by RIP were less successful. The N protein was the only virus specific protein which was successfully radiolabelled with ^{35}S-methionine and detected in RIP assays. The long exposure times required to detect this protein by autoradiography demonstrated that only low levels of incorporation of the radiolabel had been achieved. Radiolabelling was optimal when using a prolonged labelling period (about 26 h) in the presence of unlabelled methionine at levels of up to 10-15% the concentration normally present in media.

Radiolabelled N protein was detected on protein gels in cell supernates only: in cell lysates it was obscured by cellular proteins. In RIP assays the N protein occurred as a doublet of bands with MWs of 50 and 52 KD. A similar pattern has been reported by other groups (Deregt et al, 1983; Vautherot and Laporte, 1983; Keck et al, 1988). The M, S and HE gps failed to incorporate sufficient amounts of radiolabel to allow them to be detected in these assays and they may also have been obscured by cellular proteins. Other groups have encountered similar problems in detecting radiolabelled S and HE gps of BCV (Deregt et al, 1983; Keck et al, 1988).

Problems arose in the radiolabelling experiments largely because most of the radiolabel taken up by the cells was used in cell protein synthesis. Inclusion of actinomycin D at levels of up to 5 ug/ml failed to resolve this problem. Better radiolabelling might have been achieved if the virus had been grown in a cell line with a slower rate of synthesis of cell proteins. Immunofluorescent staining of acetone fixed cells and measurement of the infectivity titres of unlabelled controls demonstrated that failure to detect radiolabelled M, S and HE gps was not due to failure of virus growth. Radiolabelled N protein was probably successfully detected because it is the most abundant viral protein (King and Brian, 1982).

Shortage of time precluded further attempts to improve the radiolabelling of virus proteins. The successful labelling and immunoprecipitation of the N protein suggests the methods adopted were sound. The next step would be to purify radiolabelled virus

prior to use in RIP assays. In vitro radiolabelling of purified virus with ^{125}I could also be attempted (King and Brian, 1982). The problems of achieving successful radiolabelling can be avoided by using silver staining to detect immunoprecipitated proteins. This method is suitable when the proteins have been precipitated by MAbs but gives high levels of background staining when using polyclonal sera (Utter et al, 1986).

Epitope mapping

Interpretation of results obtained in competition ELISAs

Competition ELISAs are widely used for analysing viral antigens and for mapping biological activities to specific regions on a protein. The number of antigenic regions defined by these tests is dependent on the number of MAbs available. Best results are achieved using a large panel of MAbs which have been generated by a variety of procedures to ensure the panel is as diverse as possible. The results of competition ELISAs should be interpreted with caution as they are influenced by a number of factors.

The capture Ab and antigen preparations may influence the availability of certain epitopes, particularly because the conditions employed in ELISAs lead to some degree of antigen denaturation. The same stocks of capture Ab and antigen were therefore used throughout these tests.

Biotin/avidin was chosen as the detection system in the competition ELISAs for several reasons. Firstly, biotinylation is a simple procedure. Biotin is a relatively small molecule and is therefore less likely to affect the antigen binding site than

larger molecules such as enzymes. Biotin binds very strongly to avidin, forming complexes which are stable during subsequent procedures. The biotin/avidin system is a safer technique than the alternative method of using radiolabelled Abs (Guesdon et al, 1979; Kendall et al, 1983). Successful biotinylation of MAb S2/5 was not achieved. Alternative approaches would have been to conjugate the MAb with an enzyme such as HRP or to radioiodinate it for use in competition RIAs.

The results of competition ELISAs may be affected by variations in the levels of non-specific binding of biotinylated MAbs. Binding of biotinylated HE and S MAbs to negative control MI HRT-18 cells was negligible and was therefore ignored. The higher levels of non-specific binding which occurred with the N MAbs was allowed for in subsequent calculations. MAb isotypes and binding affinities can also affect the interpretation of results obtained in competition ELISAs. These MAbs all belonged to the IgG class of immunoglobulins and binding affinity curves demonstrated that MAbs directed against the same proteins had similar binding affinities.

Absence of competition between 2 MAbs may indicate that they are directed against different antigenic regions. MAbs directed against a single antigenic region may also fail to compete if they have widely differing binding affinities. Competition between 2 MAbs may indicate that they are directed against the same or overlapping regions, particularly if competition is reciprocal and occurs over a wide range of competitor MAb dilutions. Competition may also occur between 2 MAbs which are directed against different antigenic regions due to structural similarities in their binding

sites, steric hindrance or Ab induced conformational changes (Yewdell and Gerhard, 1981).

Epitope mapping of the HE gp

In these experiments the 4 HE MAbs all competed strongly with each other, defining a single antigenic region. This region may be subdivided into 3 sites on the basis of results obtained in other tests. MAb S2/1 was unique in its failure to bind to blotted viral proteins (Chapter 4) and in its reactions in ELISAs, IF and HAI tests with certain BCV isolates (Chapter 6). MAbs S2/3 and S2/4 were differentiated from MAbs S2/1 and S2/7 by their failure to neutralise certain virus isolates (Chapter 6). One antigenic region is therefore defined by MAb S2/1, a second by MAbs S2/3 and S2/4 and a third by MAb S2/7. The competition ELISAs demonstrate that these 3 regions are structurally or spatially closely related. The 3 epitopes (A, B and C) defined in competition ELISAs by Deregt and Babiuk (1987) were also in close spatial proximity to each other, as region B was found to overlap regions A and C. MAbs S2/1 and S2/7 probably map to antigenic region A, as they have high SN and HAI titres. MAbs S2/3 and S2/4 have lower SN and HAI titres, and may be directed against region B.

The single S MAb (S2/2) demonstrated high levels of self competition. The 4 HE MAbs also competed with this Ab, possibly because they had higher binding affinities. An alternative explanation is that binding of the HE MAbs induced conformational changes in the S gp, preventing binding of S2/2 detecting MAb (Cepica et al, 1990). This finding supports a recent proposal

made by Storz et al (personal communication) which suggested that the S gp is directly involved with haemagglutination, and that HE MAbs which cause HAI do so by a steric effect on the S gp. This suggestion was based on the finding that HE MAbs inhibit virus induced haemagglutination of chicken RBCs, but isolated HE gp cannot agglutinate RBCs from this species (Schultze et al, 1991).

Epitope mapping of the N protein

The N MAbs S2/6 and S2/8 were successfully biotinylated and used in competition ELISAs. No competition was observed between these 2 MAbs, demonstrating that they mapped to distinct antigenic sites. The HE MAbs also showed moderate levels of competition with biotinylated S2/6 detecting MAb, possibly because they had higher binding affinities. This may also have been due to steric hindrance, as the N protein is relatively small compared to the large projecting HE gps. It was not possible to test MAb S2/5 as a biotinylated detecting Ab, but the failure of this MAb to compete with MAbs S2/6 and S2/8 suggests that it is directed against a third antigenic region. This was supported by results obtained in IF tests with certain BCV isolates (Chapter 6). Three antigenic regions were thus defined on the N protein: this is the first time different antigenic regions have been demonstrated on the N protein of BCV.

Future studies: structural analysis of epitopes

Further analysis of the structural proteins of BCV is required, as their antigenic regions have so far only been partially defined

on a functional basis. A structural analysis may be achieved by several methods. One approach is to digest the virus proteins with enzymes and to detect the peptide fragments with MAbs. Alternatively, proteolysis may be performed on Ag-Ab complexes and the Ab bound fragments detected by PAGE (Deregt et al, 1989b). Antigenic structure may also be investigated by testing the binding of MAbs to synthetic peptides. These peptides are in a denatured state so MAbs directed against conformation dependent epitopes are unlikely to bind (Posthumus et al, 1990). Another common approach is to use neutralising MAbs to generate escape mutants. An antigenic map may then be constructed on a functional basis by studying the reactions of MAbs in a series of tests with the mutants. This technique has yet to be applied to BCV, but has been used to study a number of other CVs (Delmas et al, 1986; Wege et al, 1988). Sequencing of the escape mutants allows a structural definition of the epitopes. Precise structural definition of epitopes is only possible using X-ray crystallography, but so far very limited use has been made of this technique (Yewdell and Gerhard, 1981; Laver et al, 1990).

CHAPTER 6

STRAIN COMPARISON OF BOVINE CORONAVIRUSES

CHAPTER 6
STRAIN COMPARISON OF BOVINE CORONAVIRUSES

Introduction

The BCVs studied to date all belong to a single serotype. Isolates have shown only minor variations in their physicochemical properties (Dea et al, 1980b), in their behaviour with polyclonal sera in counterimmunoelectrophoresis and immunodiffusion tests (Dea et al, 1982), and in their reactions with polyclonal sera and MAbs in ELISAs, IF, SN and HAI tests (Vautherot and Laporte, 1983; Czerny and Eichhorn, 1989; Deregt et al, 1989a; El-Ghorr et al, 1989). MAbs have detected variations in both the S and HE gps in these tests. Studies undertaken so far have been limited to examining a relatively small number of viruses because of the problems associated with BCV isolation. The in vivo significance of these antigenic variations is unclear at present, but Deregt et al (1989a) found that a MAb which neutralised the parent strain of BCV in vitro failed to protect against a heterologous strain in vivo, probably due to lack of epitope conservation between the 2 strains.

Five standard strains of BCV (S1, S2, CK, M and PQ) were first examined with polyclonal sera in cross IF and SN tests, and with MAbs raised against S2 strain BCV (S2 MAbs) in IF, SN and HAI tests. In order to widen the investigation, 28 viruses were isolated from diarrhoeic calf faecal samples in TOC (TOC isolates) and screened against the MAbs in ELISAs and HAI tests. From the results of these tests, 7 viruses were chosen for adaptation to growth in HRT-18 cells (MRI BCV isolates). The S2 MAbs were

titrated against these isolates in IF, SN and HAI tests, and rabbit anti-S2 serum (serum 5317) was also titrated in SN tests. The MWs of the structural proteins of the 5 standard BCV strains and the 7 MRI isolates were then compared by separating the proteins by SDS-PAGE, blotting them onto nitrocellulose paper and probing with calf anti-S2 serum (serum 5920).

Results

Tabulation of Ab titres

Duplicate titres were obtained in all IF, SN and HAI tests, and were found to lie within 4-fold (generally 2-fold) ranges. The values given in the tables (Tables 6.1, 6.2, 6.5, 6.6, 6.7, 6.11, 6.14, 6.15, 6.16, 6.17 and 6.18) are the geometric means of these duplicate titres, rounded off to the nearest 10. Homologous titres are shown underlined. Heterologous titres which differ by more than 8-fold from homologous titres are taken to indicate the presence of antigenic variations, and these titres are shown boxed. An 8-fold difference was chosen as the level of significance because this was twice the maximum difference ever detected between duplicate tests.

Standard BCV strains: Polyclonal sera

IF and SN tests

Rabbit polyclonal sera had previously been raised by Dr. A. El-Ghorr at the MRI against the 5 standard BCV strains (S1, S2, CK, M and PQ). These were titrated by the candidate in cross IF and SN tests (Tables 6.1 and 6.2). Cross HAI tests were not performed, as the HAI titres were very low ($\leq$ 40).

Table 6.1

Mean IF titres with standard BCV strains and polyclonal sera.

Mean IF titres* with standard BCV strains and polyclonal sera

Virus	Rabbit polyclonal sera to strain :				
	S1	S2	CK	M	PQ
S1	<u>1130</u>	6400	25600	1600	25600
S2	800	<u>6400</u>	12800	1600	25600
CK	800	3200	<u>12800</u>	1600	25600
M	1130	6400	18100	<u>2260</u>	25600
PQ	800	4530	12800	1600	<u>25600</u>

* Geometric means of duplicate results, rounded off to the nearest 10.
Homologous titres are underlined.

Table 6.2

Mean SN titres with standard BCV strains and polyclonal sera.

Mean SN titres* with standard BCV strains and polyclonal sera

Virus	Rabbit polyclonal sera to strain :				
	S1	S2	CK	M	PQ
S1	<u>230</u>	910	1810	160	14480
S2	230	<u>1280</u>	5120	450	20480
CK	910	2560	<u>14480</u>	910	28960
M	640	1810	3620	<u>640</u>	14480
PQ	1810	7240	14480	640	<u>28960</u>

* Geometric means of duplicate results, rounded off to the nearest 10.
Homologous titres are underlined.

The antigenic relationships (R values) between the 5 BCV strains determined in IF and SN tests were calculated from a formula similar to that used by Archetti and Horsfall (1950):

$$R = 100 \sqrt{r1 \times r2} \quad \%$$

where r1 = heterologous titre (strain 2)/homologous titre (strain 1)

r2 = heterologous titre (strain 1)/homologous titre (strain 2)

The closer the R values to 100%, the greater the antigenic similarity between the viruses. R values < 12.5% indicate the presence of antigenic variations between 2 viruses, as this corresponds to heterologous titres which differ by more than 8-fold from homologous titres. The results shown in Tables 6.3 and 6.4 indicate that these 5 strains are serologically very closely related.

Standard BCV strains: S2 MAbs

IF tests

The 8 S2 MAbs were titrated in IF tests against the 5 standard BCV strains (Table 6.5). Strain variations were only detected with MAb S2/5 (N), which reacted to a significant titre with the parent (S2) virus only.

SN tests

The 4 HE MAbs and the single S MAb were titrated in SN tests against the 5 standard BCV strains (Table 6.6). All the MAbs neutralised all the virus strains, apart from MAbs S2/3 and S2/4 which both failed to neutralise strains CK and M (SN titres <100).

Table 6.3

The antigenic relationships (R values) of the 5 standard BCV strains as determined by IF tests.

The antigenic relationships (R values) of the 5 standard BCV strains as determined by IF tests

Virus	Rabbit polyclonal sera to strain :				
	S1	S2	CK	M	PQ
S1	100				
S2	84	100			
CK	119	71	100		
M	84	84	100	100	
PQ	84	84	100	84	100

Table 6.4

The antigenic relationships (R values) of the 5 standard BCV strains as determined by SN tests.

The antigenic relationships (R values) of the 5 standard BCV strains as determined by SN tests

Virus	Rabbit polyclonal sera to strain :				
	S1	S2	CK	M	PQ
S1	100				
S2	60	100			
CK	70	59	100		
M	83	71	60	100	
PQ	198	141	100	71	100

Table 6.5

Mean IF titres with standard BCV strains and S2 MAbs.

Mean IF titres* with standard BCV strains and S2 MAbs

Virus	Mean IF titres to MAbs : HE				S		N	
	S2/1	S2/3	S2/4	S2/7	S2/2	S2/5	S2/6	S2/8
S1	72400	51200	25600	18100	4530	<100	18100	200
S2	102400	25600	25600	18100	4530	9050	18100	140
CK	51200	18100	36200	18100	4530	<100	9050	200
M	102400	51200	25600	18100	6400	<100	25600	100
PQ	102400	25600	25600	12800	3200	<100	4530	200

* Geometric means of duplicate results, rounded off to the nearest 10. Homologous titres are underlined, and titres beyond an 8 - fold range of these titres are shown by [ ] .

Table 6.6

Mean SN titres with standard BCV strains and S2 MAbs.

Mean SN titres* with standard BCV strains and S2 MAbs

Virus	Mean SN titres to MAbs : HE				S
	S2/1	S2/3	S2/4	S2/7	S2/2
S1	4530	280	400	4530	36200
S2	6400	1600	1130	4530	6400
CK	9050	<100	<100	3200	6400
M	18100	<100	<100	3200	9050
PQ	51200	400	200	4530	1130

* Geometric means of duplicate results, rounded off to the nearest 10. Homologous titres are underlined, and titres beyond an 8 - fold range of these titres are shown by [ ].

HAI tests

The 4 HE MAbs were titrated against the 5 standard BCV strains in HAI tests (Table 6.7). No strain variations were detected, as heterologous titres were all within 8-fold ranges of the homologous (S2) virus titres.

TOC isolates: S2 MAbs

The S2 MAbs were used in ELISAs and HAI tests to screen 28 TOC isolates for strain variations.

ELISAs

The 8 MAbs were used as coating Abs to capture the test viruses in ELISAs. Chequerboard titrations of the coating MAbs and detecting Ab (rabbit anti-PQ serum, serum 5324) were first performed to find the optimal dilutions which gave OD values of about 1.0 with a positive control antigen. The optimal dilutions of the MAbs are shown in Table 6.8. Serum 5324 was used at a dilution of 1:50,000 in all tests. The specificities of the tests were confirmed by including MI TOC harvests as negative controls and by blocking positive signals with lamb anti-BCV serum, serum 5000.

The 28 TOC isolates were tested against the 8 MAbs in the ELISAs at standard HA titres of 32. Positive control wells containing S2 virus and negative control wells containing MI TOC harvests were included on all test plates. The mean OD values obtained from duplicate test and control wells are shown in Table 6.9. The first

Table 6.7

Mean HAI titres with standard BCV strains and S2 MAbs.

Mean HAI titres* with standard BCV strains and S2 MAbs

Virus	Mean HAI titres to MAbs : HE			
	S2/1	S2/3	S2/4	S2/7
S1	1280	450	640	>2560
S2	<u>5120</u>	<u>640</u>	<u>910</u>	<u>1280</u>
CK	2560	910	640	1280
M	1280	320	640	1280
PQ	2560	320	320	>2560

* Geometric means of duplicate results, rounded off to the nearest 10. Homologous titres are underlined.

Table 6.8

Dilutions of MAbs used in ELISAs

MAb	Dilution
S2/1	1:12800
S2/2	1:1000
S2/3	1:16000
S2/4	1:4000
S2/5	1:125
S2/6	1:1000
S2/7	1:8000
S2/8	1:200

Table 6.9

Mean OD values obtained in ELISAs with TOC isolates and S2 MAbs

Virus	MAbs							
	HE				S		N	
	S2/1	S2/3	S2/4	S2/7	S2/2	S2/5	S2/6	S2/8
K2595/1	0.96	1.09	1.15	1.26	0.58	0.84	1.21	0.61
K2595/2	1.13	1.10	1.23	1.32	0.60	0.91	1.27	0.68
L1096	0.98	0.73	1.10	1.24	1.01	1.19	1.30	0.67
L1121	1.04	0.98	1.23	1.30	1.09	1.48	1.40	0.50
L1209	1.00	1.13	1.26	1.37	1.07	1.49	1.47	0.61
L1217	0.93	0.85	0.97	1.16	0.21	0.73	1.07	0.68
L1280/2	0.67	0.56	0.92	1.12	0.42	0.75	0.93	0.55
L1354/2	0.83	0.88	1.03	1.17	0.51	0.70	0.99	0.55
L3080	0.75	0.82	0.97	1.11	0.15	0.68	1.03	0.58
L3140/1	0.73	0.62	0.89	1.06	0.74	1.02	1.14	0.96
L3140/2	0.73	0.75	0.92	1.07	0.46	0.62	0.92	0.70
L3140/3	0.88	0.86	1.06	1.20	0.71	1.10	1.27	0.98
L3228	1.02	1.05	1.12	1.23	0.54	0.79	1.12	0.64
L3275	1.02	1.08	1.16	1.22	0.27	1.13	1.31	0.72
L3318	0.96	1.06	1.19	1.26	0.73	1.14	1.32	0.69
L3352	0.13	1.01	1.06	1.22	0.75	0.80	1.02	0.57
L3372	0.21	0.84	1.02	1.19	0.70	0.79	1.15	0.91
L3472	0.95	0.98	1.03	1.26	0.70	0.92	1.12	1.00
S2	1.27	1.23	1.14	1.32	1.44	0.80	1.13	0.61
MI TOC	0.17	0.16	0.11	0.09	0.23	0.25	0.12	0.23

OD values obtained with the homologous virus are underlined.

Table 6.9 (continued)

Virus	MAbs							
	HE				S	N		
	S2/1	S2/3	S2/4	S2/7	S2/2	S2/5	S2/6	S2/8
N164	0.56	0.66	0.93	1.10	0.15	0.73	1.08	0.87
N225	0.22	0.80	1.02	1.15	0.59	0.84	1.09	0.46
N1193	1.09	0.99	1.15	1.34	0.80	1.13	1.26	0.74
N1334	0.32	1.12	1.21	1.37	1.14	1.26	1.31	0.89
N1258/2	1.05	1.05	1.15	1.34	0.94	1.26	1.31	0.75
0011	1.18	1.27	1.26	1.40	0.14	0.87	1.37	0.68
S2	1.27	1.23	1.14	1.32	1.44	0.80	1.13	0.61
MI TOC	0.17	0.16	0.11	0.09	0.23	0.25	0.12	0.23
L3032	0.94	0.63	1.07	1.23	0.55	1.19	1.34	0.85
N339	1.05	0.76	1.18	1.39	0.60	1.31	1.39	0.47
N539	0.78	0.80	1.26	1.45	0.65	1.21	1.47	0.93
N710	0.91	0.64	1.06	1.26	0.47	1.21	1.30	0.59
S2	1.49	0.99	1.34	1.48	1.32	1.13	1.50	0.64
MI TOC	0.18	0.10	0.10	0.09	0.17	0.29	0.14	0.24

OD values obtained with the homologous virus are underlined.

24 samples (K2591/1 to 0011) were tested on a different set of ELISA plates to the remaining 4 samples (L3032 to N710). The OD values obtained in the positive and negative control wells differed slightly between the 2 sets of plates, so the control OD values obtained with each batch of samples have been indicated in this table.

The binding of the test samples with each MAb was compared with that of the parent S2 virus by expressing the mean OD values (ODt) obtained in duplicate test wells as percentages of the mean OD values (ODs) obtained with S2 virus grown in TOC (%ODt) (Dea and Tijssen, 1989a; Taguchi and Fleming, 1989). Since there were variations in the degree of non-specific binding of MI TOC harvests to the different MAbs (ODc values = 0.09 - 0.29), the mean ODc values were subtracted from the OD values used to calculate the % ODt values. The final equation was therefore:

$$\%ODt = \frac{(ODt - ODc)}{(ODs - ODc)} \times 100$$

Where ODt = mean OD with test sample

ODc = mean OD with MI TOC harvest

ODs = mean OD with S2 virus

The calculated %ODt values are given in Table 6.10, and the number of TOC isolates having %ODt values within appropriate ranges are shown in Figs. 6.1 and 6.2. %ODt values <25% were taken to indicate the presence of significant antigenic variations from S2 virus. This cut off value had previously been used by Dea and Tijssen (1989a) during a series of similar ELISAs designed to

Table 6.10

% OD values* obtained in ELISAs with TOC isolates and S2 MAbs

Virus	MAbs							
	HE				S	N		
	S2/1	S2/3	S2/4	S2/7	S2/2	S2/5	S2/6	S2/8
K2595/1	72	86	101	94	29	106	107	99
K2595/2	87	87	109	100	31	119	114	117
L1096	73	53	96	93	65	170	117	114
L1121	79	76	108	98	71	224	127	70
L1209	76	90	111	103	70	226	133	100
L1217	69	64	84	87	0	87	94	118
L1280/2	46	37	79	83	16	90	80	84
L1354/2	60	66	89	87	23	81	86	83
L3032	58	59	79	82	33	107	89	151
L3080	52	61	83	83	0	78	90	91
L3140/1	51	43	75	78	42	139	100	192
L3140/2	56	55	79	79	19	66	79	122
L3140/3	64	65	92	90	39	154	114	197
L3228	77	83	97	92	26	98	99	107
L3275	77	85	102	91	3	159	118	129
L3318	72	84	104	95	41	162	119	121
L3352	0	79	92	91	43	100	89	88
L3372	4	63	88	89	39	98	97	178
L3472	71	76	89	95	39	121	99	203
N164	35	47	79	82	0	87	95	167

Table 6.10 (continued)

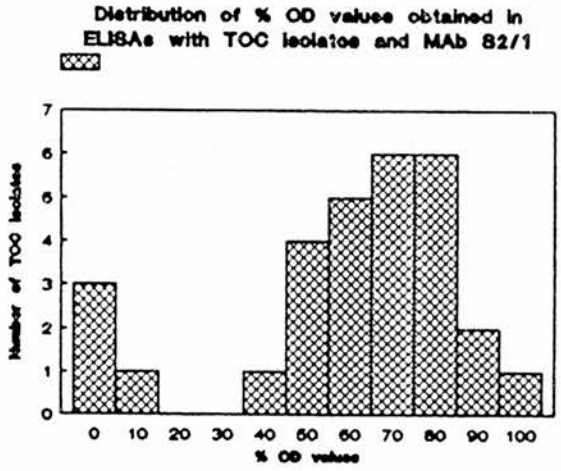
Virus	MAbs							
	HE				S	N		
	S2/1	S2/3	S2/4	S2/7	S2/2	S2/5	S2/6	S2/8
N225	[4]	60	88	85	29	107	96	59
N339	66	73	87	93	37	121	92	56
N539	46	78	95	98	41	109	98	173
N710	55	60	78	84	26	109	85	88
N1193	83	77	100	101	47	159	112	134
N1334	[14]	89	106	104	76	183	117	174
N1258/2	80	83	101	101	59	183	118	137
0011	92	104	111	106	0	112	123	117
S2	100	100	100	100	100	100	100	100

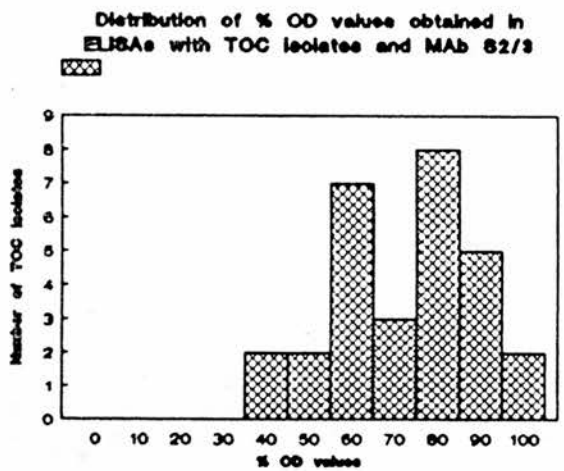
* % OD values given by : $\frac{ODt - ODc}{ODs - ODc} \times 100$ Where ODt = mean OD with test sample
ODc = mean OD with MI TOC
ODs = mean OD with S2 virus

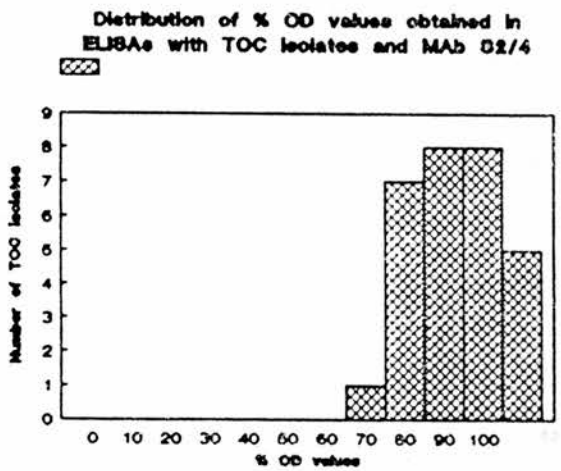
For the HE and N MAbs, % OD values < 25% are shown by : [ ] .

Figure 6.1

Distribution of %ODt values obtained in ELISAs with TOC isolates and MAbs S2/1, S2/3, S2/4 and S2/7.


Distribution of % OD values obtained in ELISAs with TOC isolates and MAb S2/1
Number of TOC isolates
0 1 2 3 4 5 6 7
0 10 20 30 40 50 60 70 80 90 100
% OD values


Distribution of % OD values obtained in ELISAs with TOC isolates and MAb S2/3
Number of TOC isolates
0 1 2 3 4 5 6 7 8 9
0 10 20 30 40 50 60 70 80 90 100
% OD values


Distribution of % OD values obtained in ELISAs with TOC isolates and MAb S2/4
Number of TOC isolates
0 1 2 3 4 5 6 7 8 9
0 10 20 30 40 50 60 70 80 90 100
% OD values

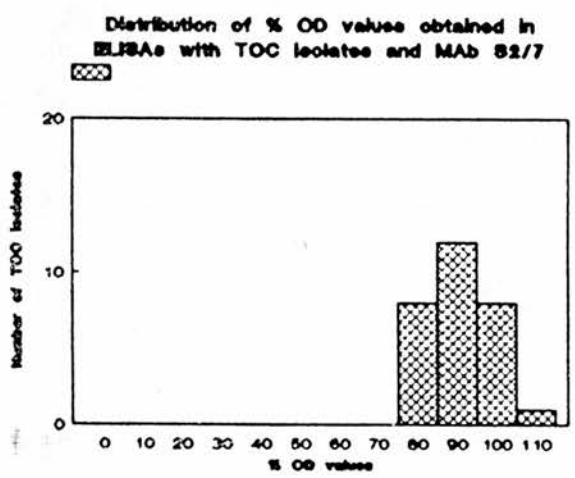
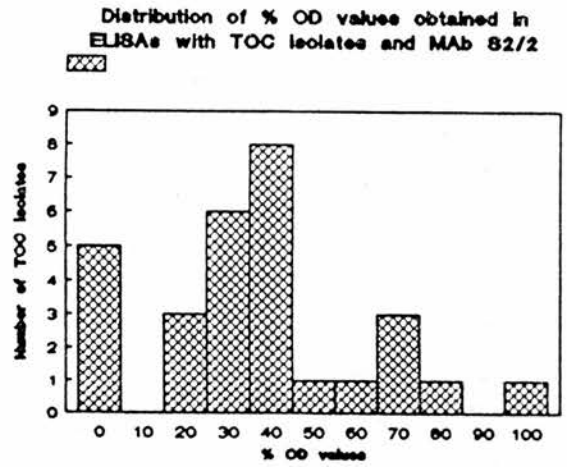
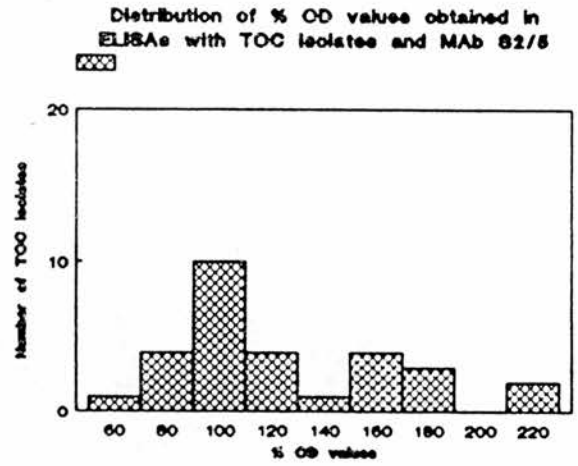
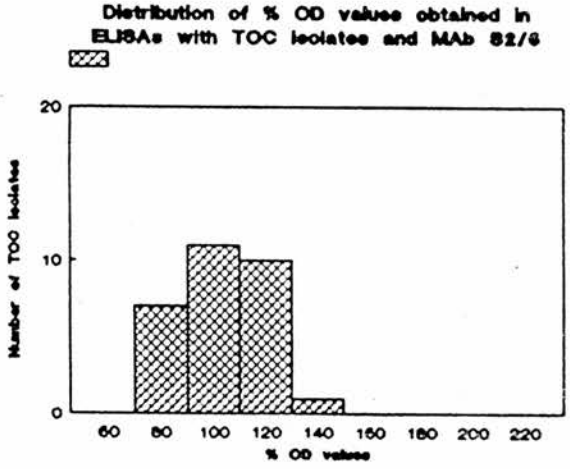

Distribution of % OD values obtained in ELISAs with TOC isolates and MAb S2/7
Number of TOC isolates
0 10 20
0 10 20 30 40 50 60 70 80 90 100 110
% OD values

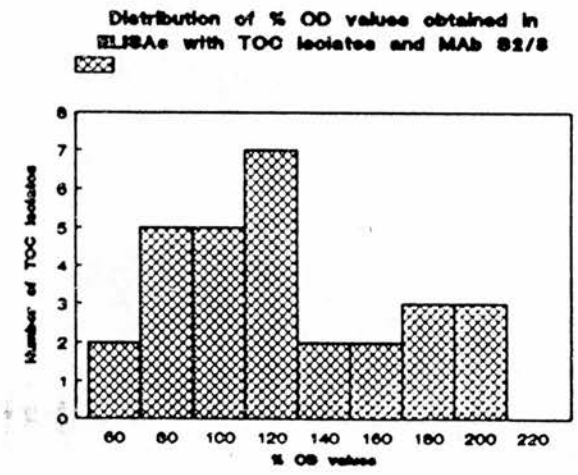
Figure 6.2

Distribution of %ODt values obtained in ELISAs with TOC isolates and MAbs S2/2, S2/5, S2/6 and S2/8.


Distribution of % OD values obtained in ELISAs with TOC isolates and MAb S2/2
Number of TOC isolates
0 1 2 3 4 5 6 7 8 9
0 10 20 30 40 50 60 70 80 90 100
% OD values


Distribution of % OD values obtained in ELISAs with TOC isolates and MAb S2/5
Number of TOC isolates
0 10 20
60 80 100 120 140 160 180 200 220
% OD values


Distribution of % OD values obtained in ELISAs with TOC isolates and MAb S2/6
Number of TOC isolates
0 10 20
60 80 100 120 140 160 180 200 220
% OD values


Distribution of % OD values obtained in ELISAs with TOC isolates and MAb S2/8
Number of TOC isolates
0 1 2 3 4 5 6 7 8
60 80 100 120 140 160 180 200 220
% OD values

investigate the extent of antigenic variation amongst TCVs. The results obtained with MAb S2/1 clearly demonstrate that this cut off value was also appropriate to these studies, as it defined 2 distinct populations of viruses. The majority of TOC isolates bound strongly to this MAb giving relatively high %ODt values, whilst 4 isolates either failed to bind or only bound very weakly to this MAb (%ODt values $\leq$ 14). The other 3 HE MAbs (S2/3, S2/4 and S2/7) and the 3 N MAbs (S2/5, S2/6 and S2/8) failed to detect antigenic variations amongst the TOC isolates. The results obtained with MAb S2/2 were more difficult to interpret, as a wide range of %ODt values were obtained which failed to define distinct populations of viruses. For this MAb, it was clearly inappropriate to take <25% as a cut off value indicative of the presence of antigenic variation.

HAI tests

Ascitic fluids containing the 4 HE MAbs (S2/1, S2/3, S2/4 and S2/7) were titrated against 8 HA units of each of the TOC isolates. The geometric mean titres obtained from duplicate wells are shown in Table 6.11. MAb S2/8 (N) was used as a negative control: at a dilution of 1:10 it failed to inhibit the haemagglutination caused by any of the test viruses. S2 virus grown in TOC was used as a positive control. HAI titres which lay beyond an 8-fold range of those obtained with S2 virus were taken to indicate the presence of antigenic variations. MAbs S2/3 and S2/4 failed to detect any strain variations, but 7 viruses reacted weakly with MAb S2/1, 2 of which also reacted weakly with MAb S2/7.

Table 6.11

Mean HAI titres* with TOC isolates and S2 MAbs

Virus	Mean HAI titres to MAbs : HE			
	S2/1	S2/3	S2/4	S2/7
K2595/1	160	450	450	160
K2595/2	450	1280	640	450
L1096	320	450	320	230
L1121	320	640	1280	910
L1209	160	640	450	230
L1217	160	640	640	160
L1280/2	[30]	320	320	[60]
L1354/2	80	640	640	160
L3032	450	640	640	450
L3080	640	1280	1280	640
L3140/1	230	640	640	320
L3140/2	320	910	640	320
L3140/3	640	640	640	450
L3228	160	450	640	160
L3275	230	910	640	450
L3318	320	2560	1280	1280
L3352	[30]	910	910	450
L3372	[30]	640	450	230

Table 6.11 (continued)

Virus	Mean HAI titres to MAbs : HE			
	S2/1	S2/3	S2/4	S2/7
L3472	910	1280	1280	640
N164	[30]	640	640	[110]
N225	[40]	1280	1280	640
N339	320	910	910	640
N539	160	2560	2560	910
N710	450	910	640	450
N1193	640	1280	640	640
N1334	[30]	640	640	450
N1258/2	230	1280	1280	320
0011	[<10]	1280	910	640
S2	640	640	640	1280

*Geometric means of duplicate results, rounded off to the nearest 10.

Homologous titres are underlined, and titres > 8-fold different from the homologous titres are shown by [ ] .

On the basis of the results obtained in the ELISAs and HAI tests, 7 of the TOC isolates were chosen for adaptation to growth in HRT-18 cells (Chapter 3). These viruses were then referred to as the MRI BCV isolates.

MRI BCV isolates: S2 MAbs

ELISAs

The 7 MRI BCV isolates were tested against the 8 S2 MAbs in ELISAs at standard HA titres of 32. The mean OD values obtained from duplicate test and control wells are shown in Table 6.12. The viruses were again tested in 2 batches, so the control OD values obtained with each batch are indicated on the table. These results were then used to calculate the %ODt values shown in Table 6.13, and these have been plotted as histograms in Figs 6.3 and 6.4. The results were similar to those previously obtained using TOC harvests. MAb S2/1 again defined 2 distinct populations of virus, with 3 viruses (L3352, L3372 and N225) reacting weakly with this MAb. The other 3 HE MAbs and the 3N MAbs failed to detect antigenic variations. A wide range of %ODt values were obtained with MAb S2/2, but a clear cut off value could not be defined.

IF tests

The 8 S2 MAbs were titrated against the 7 MRI BCV isolates in IF tests (Table 6.14). The results were consistent with those obtained in the ELISAs. Three viruses (L3352, L3372 and N225) reacted weakly with MAb S2/1 (HE) and 2 viruses (N164 and 0011) reacted weakly

Table 6.12

Mean OD values obtained in ELISAs with MRI BCV isolates and S2 MAbs

Virus	MAbs							
	HE				S	N		
	S2/1	S2/3	S2/4	S2/7	S2/2	S2/5	S2/6	S2/8
L3352	0.13	1.24	1.56	1.50	1.01	1.18	1.50	0.73
L3372	0.14	1.33	1.65	1.56	1.20	1.21	1.69	0.79
N539	0.80	1.05	1.39	1.49	0.62	0.48	1.08	0.71
0011	0.72	1.06	1.43	1.63	0.33	0.53	1.41	0.64
S2	1.21	1.53	1.54	1.66	1.58	0.86	1.34	0.85
MI HRT-18 cells	0.08	0.10	0.08	0.08	0.19	0.17	0.15	0.16
N225	0.05	0.43	0.62	0.62	0.40	0.46	0.74	0.18
L3080	0.39	0.49	0.67	0.62	0.19	0.37	0.69	0.20
N164	0.34	0.39	0.49	0.50	0.15	0.34	0.58	0.20
S2	0.48	0.57	0.66	0.63	0.55	0.23	0.54	0.29
MI HRT-18 cells	0.02	0.02	0.01	0.02	0.04	0.01	0.02	0.02

OD values obtained with the homologous virus are underlined.

Table 6.13

% OD values* obtained in ELISAs with MRI BCV isolates and S2 MAbs

Virus	MAbs							
	HE				S	N		
	S2/1	S2/3	S2/4	S2/7	S2/2	S2/5	S2/6	S2/8
L3352	4	79	101	90	59	93	113	83
L3372	5	86	108	94	72	151	129	91
N225	7	75	94	98	71	205	136	65
N539	63	66	90	89	31	45	78	80
L3080	80	85	102	98	29	164	127	71
N164	68	67	74	80	22	150	107	69
0011	56	67	92	98	10	51	106	69
S2	100	100	100	100	100	100	100	100

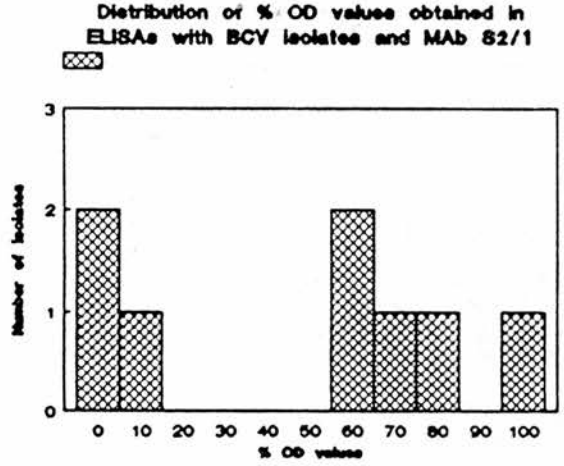
* % OD values given by : $\frac{ODt - ODc}{ODs - ODc} \times 100$

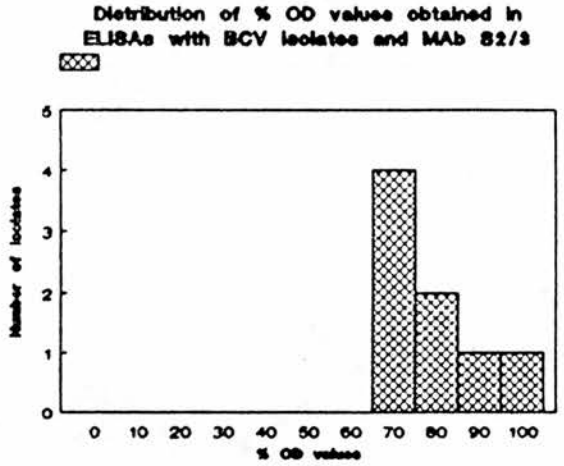
Where OD t = mean OD with test sample
ODc = mean OD with MI HRT-18 cells
ODs = mean OD with S2 virus

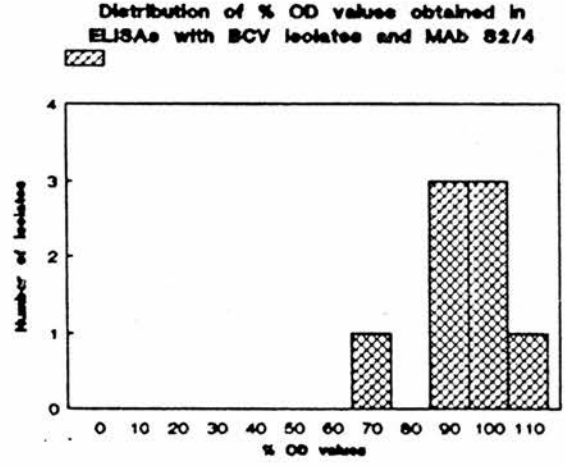
For the HE and N MAbs, % OD values < 25 % are shown by : [ ] .

Figure 6.3

Distribution of %ODt values obtained in ELISAs with MRI BCV isolates and MAbs S2/1, S2/3, S2/4 and S2/7.


Distribution of % OD values obtained in ELISAs with BCV isolates and MAb S2/1
Number of isolates
0
1
2
3
0 10 20 30 40 50 60 70 80 90 100
% OD values


Distribution of % OD values obtained in ELISAs with BCV isolates and MAb S2/3
Number of isolates
0
1
2
3
4
5
0 10 20 30 40 50 60 70 80 90 100
% OD values


Distribution of % OD values obtained in ELISAs with BCV isolates and MAb S2/4
Number of isolates
0
1
2
3
4
0 10 20 30 40 50 60 70 80 90 100 110
% OD values

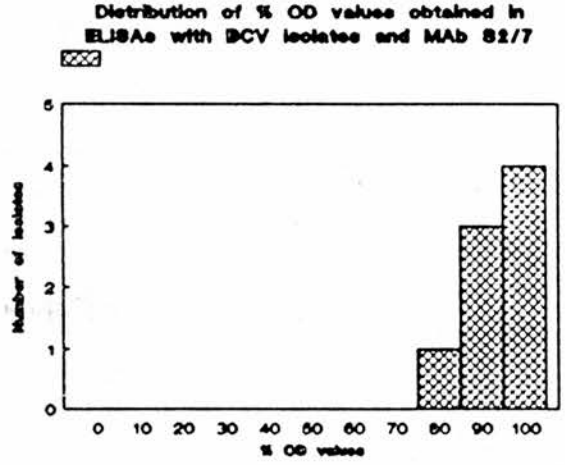
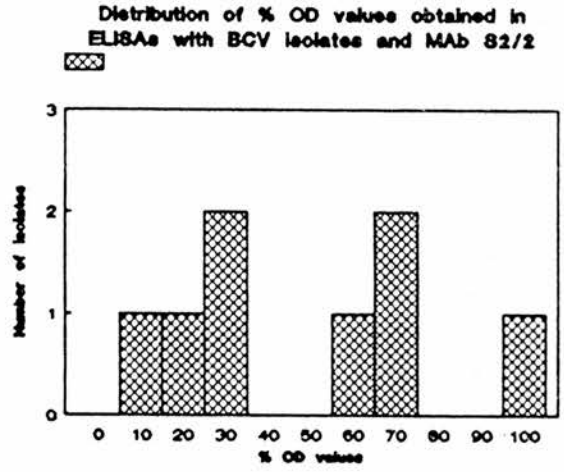
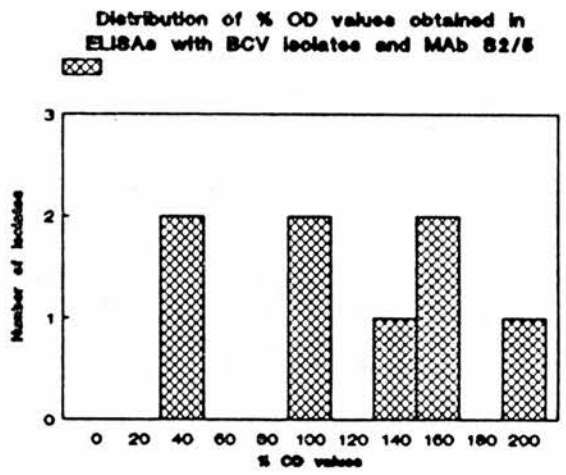
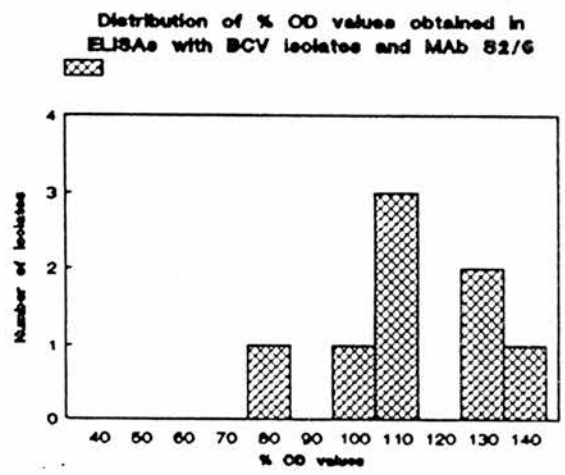

Distribution of % OD values obtained in ELISAs with BCV isolates and MAb S2/7
Number of isolates
0
1
2
3
4
5
0 10 20 30 40 50 60 70 80 90 100
% OD values

Figure 6.4

Distribution of %ODt values obtained in ELISAs with MRI BCV isolates and MAbs S2/2, S2/5, S2/6 and S2/8.


Distribution of % OD values obtained in ELISAs with BCV isolates and MAb S2/2
Number of isolates
0
1
2
3
0 10 20 30 40 50 60 70 80 90 100
% OD values


Distribution of % OD values obtained in ELISAs with BCV isolates and MAb S2/5
Number of isolates
0
1
2
3
0 20 40 60 80 100 120 140 160 180 200
% OD values


Distribution of % OD values obtained in ELISAs with BCV isolates and MAb S2/6
Number of isolates
0
1
2
3
4
40 50 60 70 80 90 100 110 120 130 140
% OD values

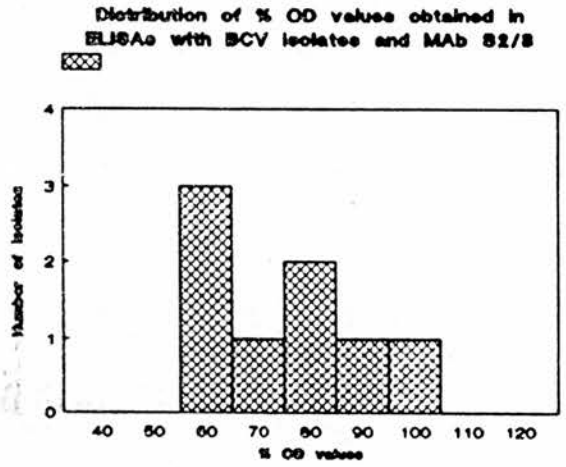

Distribution of % OD values obtained in ELISAs with BCV isolates and MAb S2/8
Number of isolates
0
1
2
3
4
40 50 60 70 80 90 100 110 120
% OD values

Table 6.14

Mean IF titres with MRI BCV isolates and S2 MAbs.

Mean IF titres* with MRI BCV isolates and S2 MAbs

Virus	Mean IF titres to MAbs : HE				S	N		
	S2/1	S2/3	S2/4	S2/7	S2/2	S2/5	S2/6	S2/8
L3352	1600	51200	18100	>12800	>12800	18100	>12800	200
L3372	4530	51200	25600	>12800	>12800	12800	>12800	140
N225	1130	102400	51200	25600	6400	25600	>12800	200
N539	>102400	51200	25600	>12800	>12800	25600	>12800	200
L3080	>102400	>102400	51200	25600	570	25600	>12800	280
N164	>102400	>102400	51200	12800	<50	12800	36200	200
OO11	>102400	>102400	51200	>12800	400	25600	>12800	200
S2	102400	25600	25600	18100	4530	9050	18100	140

* Geometric means of duplicate results, rounded off to the nearest 10.

Homologous titres are underlined, and titres beyond an 8 - fold range of these titres are shown by [ ].

with MAb S2/2 (S). The titre obtained with MAb S2/2 and L3080 was on the edge of the previously defined limit of significance.

HAI tests

The 4 MAbs directed against the HE gp of S2 strain BCV were titrated against the 7 MRI isolates in HAI tests (Table 6.15). The ascitic fluids containing MAbs S2/1, S2/4 and S2/7 were from different stocks to those previously used in Table 6.7: the titres reported for S2 virus are therefore different. Strain variations were only detected in these tests by MAb S2/1, which reacted to low titres with L3352, L3372 and N225. These results were consistent with those obtained in ELISAs and IF tests.

SN tests

The 5 MAbs directed against the HE and S gps of S2 strain BCV were titrated against the 7 MRI isolates in SN tests (Table 6.16). The 4 HE MAbs distinguished all the MRI isolates from S2 strain virus, and 3 viruses (L3080, N164 and 0011) were also distinguished by the S MAb (S2/2).

MRI BCV isolates: Polyclonal sera

SN tests

Rabbit anti-S2 serum (serum 5317) was titrated against the MRI BCV isolates in SN tests (Table 6.17). Strain variations were not detected with this polyclonal serum, as the SN titres obtained with the heterologous isolates all lay within an 8-fold range of that obtained with the homologous (S2) virus.

Table 6.15

Mean HAI titres with MRI BCV isolates and S2 MAbs.

Mean HAI titres* with MRI BCV isolates and S2 MAbs

Virus	Mean HAI titres to MAbs : HE			
	S2/1	S2/3	S2/4	S2/7
L3352	60	450	1280	1280
L3372	40	230	1810	450
N225	80	110	320	80
N539	640	1810	1280	640
L3080	450	230	910	450
N164	2560	230	160	640
OO11	230	450	450	640
S2	910	640	640	640

* Geometric means of duplicate results, rounded off to the nearest 10.

Homologous titres are underlined, and titres beyond an 8 - fold range of these titres are shown by ☐ .

Table 6.16

Mean SN titres with MRI BCV isolates and S2 MAbs.

Mean SN titres* with MRI BCV isolates and S2 MAbs

Virus	Mean SN titres to MAbs : HE				S
	S2/1	S2/3	S2/4	S2/7	S2/2
L3352	<50	<50	<50	<50	2260
L3372	<50	<50	<50	<50	2260
N225	<50	<50	<50	<50	3200
N539	200	<50	<50	<50	2260
L3080	<50	<50	<50	<50	<50
N164	140	<50	<50	<50	<50
OO11	400	<50	<50	280	<50
S2	6400	1600	1130	4530	6400

* Geometric means of duplicate results, rounded off to the nearest 10.
Homologous titres are underlined, and titres beyond an 8-fold range of these titres are shown by [ ].

Table 6.17

Mean SN titres*

with MRI BCV isolates and rabbit anti - S2 serum

Virus	Mean SN titre
L3352	160
L3372	320
N225	320
N539	320
L3080	230
N164	320
OO11	230
S2	<u>1280</u>

*Geometric means of duplicate results, rounded off to the nearest 10.

The homologous titre is underlined.

Standard BCV strains and MRI isolates: CVL MAbs

IF tests

The MAbs directed against BCV strain CVL were titrated in IF tests against the 5 standard BCV strains and 4 of the MRI isolates (Table 6.18). The titres obtained were difficult to interpret because the parent virus was not available for comparison. The maximum variations in the titres obtained with MAbs 5528 (M), 5529 (S), 5530 (S) and 5531 (HE) were 6, 8, 11 and 64-fold respectively. This strongly suggests that MAb 5531 (HE) at least is detecting antigenic variations amongst these isolates.

Western blotting

Ultracentrifuge pellets of the 5 standard strains of BCV and the 7 MRI isolates were prepared. The infectivity (IT) and HA titres were measured and the protein concentrations estimated using a Pierce BCA Protein Assay Kit (Table 6.19). The results demonstrate that the preparations showed some variation in the amounts of virus present, particularly the S1 and L3080 pellets which had low infectivity and HA titres.

30 ul of each pellet were boiled for 90 sec with 30 ul 2 x LSB (no ME). The proteins were separated by SDS/PAGE and electrophoretically transferred to nitrocellulose paper. Staining of the blotted proteins with Ponceau S revealed many bands (Fig. 6.5). Efficient protein transfer had occurred as bands stained on the gel with Coomassie Brilliant Blue were extremely faint. Blotted proteins from the S1 virus preparation stained particularly darkly with Ponceau S, presumably due to a high concentration of cellular proteins.

Table 6.18

Mean IF titres with standard BCV strains or MRI isolates and CVL MAbs.

Mean IF* titres with standard BCV strains or MRI isolates and CVL MAbs

Virus	Mean IF titres to MAbs : M	S		HE
	5528	5529	5530	5531
S1	400	9050	12800	3200
S2	2260	18100	18100	12800
CK	1600	6400	4530	25600
M	400	6400	6400	25600
PQ	1130	12800	6400	1600
L3352	800	25600	51200	>102400
L3372	400	25600	25600	>102400
N539	800	12800	25600	51200
OO11	800	3200	6400	25600

* Geometric means of duplicate results, rounded off to the nearest 10.

Table 6.19

Analysis of virus pellets used for Western blotting

Virus	Protein concentration (ug/ml)	IT*	HA titre
S2	960	8.8	51200
S1	2135	5.8	800
M	1130	7.8	25600
PQ	2015	8.8	12800
CK	3100	6.8	12800
L3352	1440	7.3	12800
L3372	1430	7.3	6400
OO11	1080	7.3	6400
N539	2185	7.8	12800
N225	2425	6.8	12800
N164	2040	7.3	12800
L3080	1460	6.3	3200

* IT measured as log TCID50 / ml.

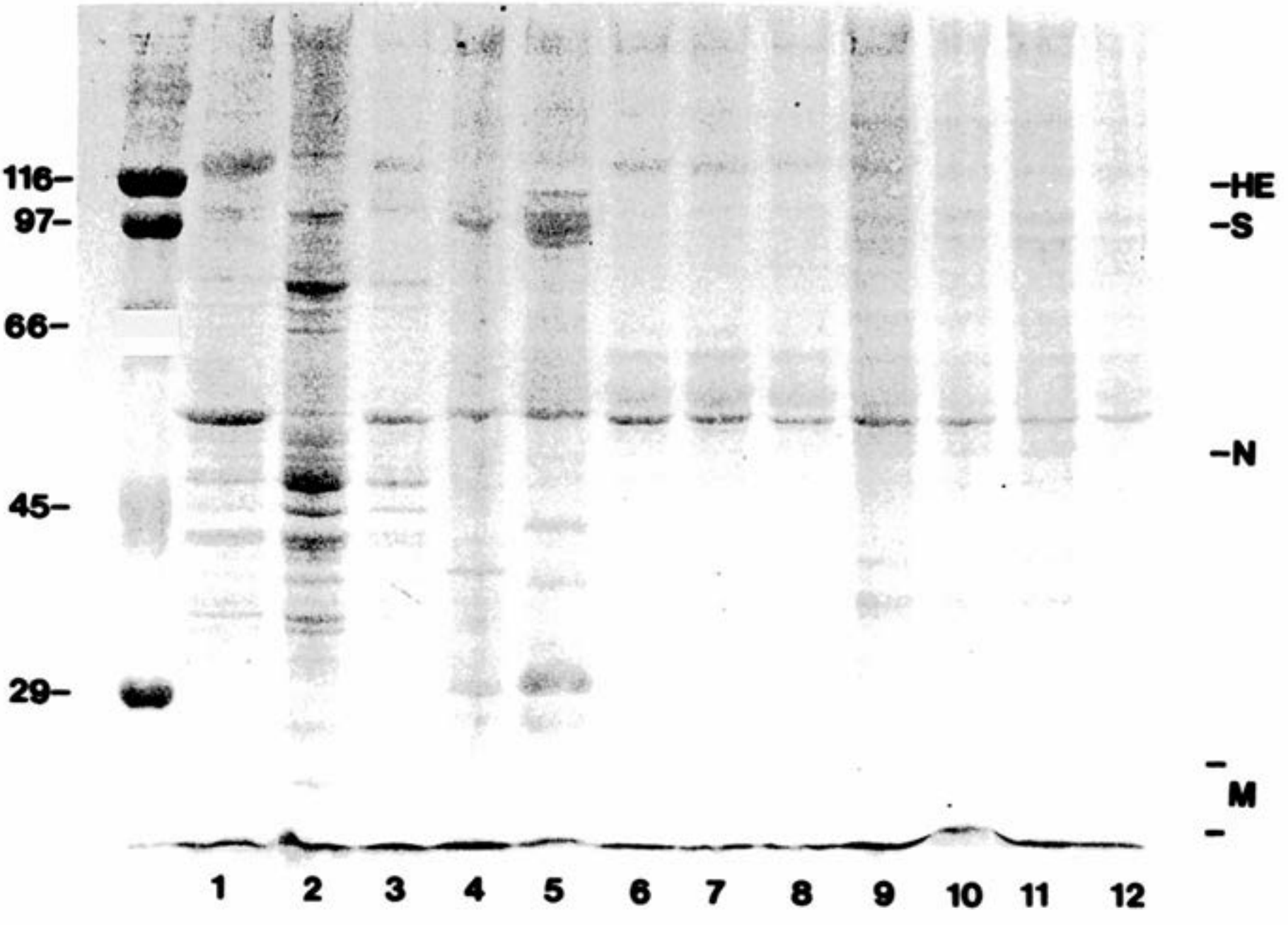
Figure 6.5

Comparison of 12 isolates of BCV by Western blotting: Blotted proteins stained with Ponceau S (non-reducing conditions)

The virus proteins were separated by SDS/PAGE and electroblotted onto nitrocellulose paper. Blotted proteins were stained with Ponceau S.

Template

Lane	Virus
1	S2
2	S1
3	M
4	PQ
5	CK
6	L3352
7	L3372
8	0011
9	N539
10	N225
11	N164
12	L3080


116–
97–
66–
45–
29–
–HE
–S
–N
M
1
2
3
4
5
6
7
8
9
10
11
12

The viral proteins were probed with gnotobiotic calf anti-S2 serum (serum 5920) at a dilution of 1:4. Bound Abs were detected with anti-sheep HRP conjugate and 4-chloro-1-naphthol substrate solution (Fig. 6.6). Bands corresponding to the HE, S, N and M proteins of the majority of virus isolates were clearly visible after probing. No bands were detected with a PBS negative control probe (data not shown). This experiment confirms that the proteins of the heterologous viruses are antigenically related to those of S2 virus. The MWs of the HE, N and M proteins of the 13 viruses were very similar. Slight variations in the MWs of the S gps were apparent, but these may have been artefacts of protein separation. Shortage of time precluded probing blotted viral proteins with the MAbs.

Discussion

Antigenic variations amongst virus isolates are most efficiently detected using a combination of polyclonal sera and MAbs. Polyclonal sera detect broad differences between viruses and MAbs detect minor antigenic variations. The results obtained using MAbs should be interpreted with caution. They may emphasise antigenic similarities between viruses which are otherwise largely dissimilar and they may detect minor variations which are of no relevance *in vivo*.

Standard BCV strains

Polyclonal sera raised against the 5 standard BCV strains failed

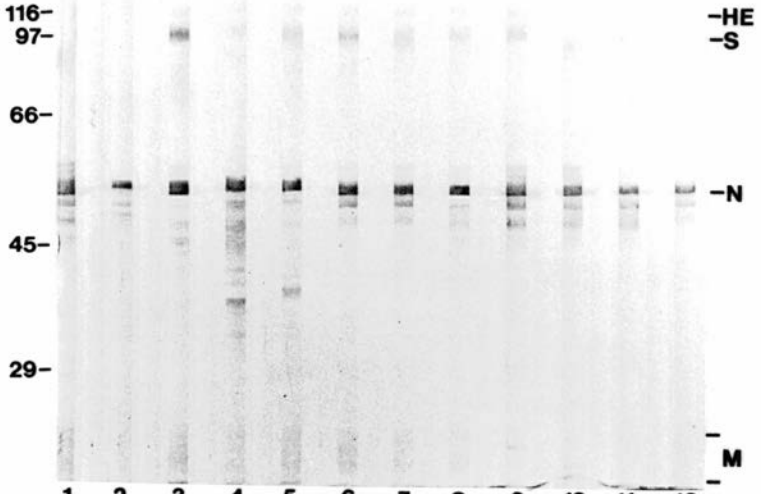
Figure 6.6

Comparison of 12 isolates of BCV by Western blotting: Virus proteins probed with gnotobiotic calf anti-S2 serum
(non-reducing conditions)

The virus proteins were separated by SDS/PAGE and electroblotted onto nitrocellulose paper. Blotted proteins were probed with gnotobiotic calf anti-S2 serum (serum 5920) and bound Abs detected with anti-sheep HRP conjugate.

Template

Lane	Virus
1	S2
2	S1
3	M
4	PQ
5	CK
6	L3352
7	L3372
8	0011
9	N539
10	N225
11	N164
12	L3080


116–
97–
66–
45–
29–
–HE
–S
–N
M
1
2
3
4
5
6
7
8
9
10
11
12

to detect antigenic variations in cross IF and SN tests. MAbs raised against S2 virus detected antigenic differences on the HE gp in SN tests (MAbs S2/3 and S2/4 failed to neutralise strains CK and M) and on the N protein in IF tests (MAb S2/5 only reacted with the parent S2 virus). This is the first time that antigenic variations have been reported on the N protein of BCV.

TOC isolates

The TOC isolates were first screened for antigenic variations using the S2 MAbs as coating Abs in ELISAs. Polyclonal rabbit anti-PQ serum (serum 5324) was chosen as the detecting Ab for all tests. None of the TOC isolates gave low OD readings with all the MAbs, which demonstrated that this polyclonal serum bound adequately to all the test viruses. Low %ODt values were therefore indicative of variations detected by the MAbs.

The viruses were standardised in the ELISAs by testing them all at the same HA titre. This method was chosen as measurement of HA titres was the simplest means of quantifying the viruses present in TOC harvests. At the time these experiments were performed, this was considered to be appropriate for the HE MAbs but less appropriate for the S and N MAbs. Since then, questions have been raised as to whether the HE gp is indeed the viral haemagglutinin, or whether the S gp is responsible for haemagglutination (Storz et al, personal communication). The method of standardisation may account for the rather low %ODt values obtained with MAb S2/2 (S) and the high (often >100) values obtained with the N MAbs. Seven of the TOC isolates were later adapted to growth in HRT-18 cells

(MRI BCV isolates). These viruses gave very similar results when tested in ELISAs and IF tests, which suggests that adequate standardisation of the ELISAs had in fact been achieved.

Antigenic variations were detected in the ELISAs by MAbs S2/1 and S2/2 only. Two clear populations of viruses were defined by MAb S2/1, as either moderate to high (>35) or very low (≤14) %ODt values were obtained. This suggests that the epitope recognised by this MAb is either present or absent on these viruses. The results obtained with MAb S2/2 are harder to interpret, as a wide range of %ODt values were obtained with no clear cut off point. These results suggest that the epitope recognised by this MAb shows a range of variation amongst different virus isolates. An alternative explanation is that the fragile peplomers were lost to a greater extent from some of the viruses.

The TOC isolates were also screened against the S2 MAbs in HAI tests, and the results were similar to those obtained in the ELISAs. The 4 TOC isolates (L3352, L3372, N225, N1334) which reacted weakly with MAb S2/1 in ELISAs also reacted weakly with this MAb in HAI tests. Three viruses (L1280/2, N164 and 0011) reacted moderately or strongly with MAb S2/1 in ELISAs but gave only weak reactions with this MAb in HAI tests. Two of these viruses, L1280/2 and N164, also reacted strongly with MAb S2/7 in ELISAs but gave only weak reactions with this MAb in HAI tests. Both MAbs S2/1 and S2/7 reacted to high HAI titres with 2 of these viruses (N164 and 0011) after they had been adapted to growth in cell culture. The low HAI titres obtained with the TOC harvests may have been artefacts caused by the presence of high levels of

subviral components or non-specific haemagglutinins. An alternative explanation is that the viruses were modified by adaptation to cell culture.

MRI BCV isolates

The MAbs behaved very similarly with the MRI isolates in ELISAs, IF and HAI tests (Table 6.20). Three viruses (L3352, L3372 and N225) reacted weakly with MAb S2/1 in all 3 tests and another 3 viruses (L3080, N164 and 0011) reacted weakly with MAb S2/2 in ELISAs and IF tests. SN tests were the most sensitive method for detecting antigenic variations. Four of the MRI isolates (L3352, L3372, N225 and N539) were strongly neutralised by the S MAb (S2/2) but were not neutralised by the HE MAbs. The remaining 3 MRI isolates (L3080, N164 and 0011) were not significantly neutralised by any of the MAbs. These tests demonstrate that the former group of viruses differ antigenically from S2 strain virus in their HE gp whilst the latter group of viruses differ in both their HE and S gps.

Comparison of the results obtained in ELISAs and IF tests with those obtained in SN tests demonstrates that the HE MAbs can bind to virus particles without neutralising them. This phenomenon has also been reported by Iorio and Bratt (1984). In contrast to the HE MAbs, the S MAb (S2/2) only bound in ELISAs and IF tests to virus isolates which it also neutralised (Table 6.20). There are several possible explanations to account for why the HE MAbs could bind to certain virus isolates whilst failing to neutralise them. The epitopes to which these MAbs bound may have had slight

Table 6.20

Summary of results of IF,HAI and SN tests with MRI BCV isolates and S2 MAbs

This table only includes the MAbs which detected antigenic variations

+ = titres within 8 - fold range of S2 titre
- = titres beyond 8 - fold range of S2 titre

Test		IF		HAI	SN	
MAb		HE S2/1	S HE S2/2	HE S2/1	HE S2/1 S2/3 S2/4 S2/7	S S2/2
Virus	L3352	-	+	-	-	+
	L3372	-	+	-	-	+
	N225	-	+	-	-	+
	N539	+	+	+	-	+
	L3080	+	+	+	-	-
	N164	+	-	+	-	-
	OO11	+	-	+	-	-

structural alterations or been present in reduced numbers in these virus isolates, thereby preventing binding of sufficient levels of MAb to cause neutralisation. If neutralisation was mediated by binding of Abs to cell receptor sites on virus particles, failure to neutralise may have been caused by the MAbs binding to sites other than the cell receptor sites. Alternatively, if neutralisation was mediated by an Ab induced change in virus surface configuration, failure to neutralise may have been caused by failure of bound Ab to initiate the appropriate structural changes.

Rabbit anti-S2 serum (serum 5317) failed to detect antigenic variations amongst the 7 MRI isolates in SN tests. This suggests that the antigenic differences detected by the MAbs may not be relevant when using polyclonal sera, and may not therefore be important in vivo. Shortage of time precluded purifying these viruses and raising polyclonal sera for use in cross SN tests.

Conclusions

This is the most comprehensive investigation of BCV strain variation which has been undertaken to date. The results support those obtained by other groups: strain variations do occur, but they are not sufficiently great as to suggest the existence of more than one serotype of the virus (Vautherot and Laporte, 1983; El-Ghorr et al, 1989). In these experiments strain variations were never detected with polyclonal sera in IF and SN tests, and extensive cross reactions occurred when gnotobiotic calf anti-S2 serum (serum 5920) was used to probe blotted viral proteins of heterologous isolates.

MAbs raised against S2 strain BCV detected antigenic variations on the N, S and HE proteins. Antigenic variations were only detected on the N protein of standard strains S1, CK, M and PQ, and not on any of the TOC or MRI BCV isolates. It may have been significant that the latter viruses were all isolated from faecal samples collected in Scotland between 1987 and 1989. In contrast, whilst S1 strain was also isolated in Scotland, strains CK, M and PQ originated from England, America and Canada respectively, and these strains were all isolated from faecal samples collected before 1987.

MAbs detected wide antigenic variations on the S and HE gps of BCV isolates. These proteins are likely to vary more than the N protein, as the host's immunological responses exert a greater selection pressure on proteins bearing neutralising epitopes. Antigenic variations were more commonly detected on the MRI isolates than on the standard BCV strains. This was not unexpected as the MRI isolates had been selected from a large number of TOC isolates on the basis of results obtained in ELISAs and HAI tests.

The significance of the antigenic variations detected by the MAbs is unclear at present. Antigenic variations which are only detected using MAbs can be important clinically. The CVs TGEV and PRCV are indistinguishable with polyclonal sera but may be differentiated with a MAb (Callebaut et al, 1989). Some field and vaccine strains of rabies virus are only distinguished using MAbs: these antigenic variations may be responsible for apparent vaccine breakdowns (Yewdell and Gerhard, 1981). The variations detected amongst the BCV isolates may affect viral pathogenicity (virulence

and organ tropism) and may explain the occurrence of the different clinical syndromes associated with BCV. It is important to establish whether heterotypic immunity occurs between different BCV strains as this affects the choice of antigens for vaccine production.

Any study of strain variation is limited by the number of virus isolates and the availability of polyclonal sera and MAbs. In these experiments all the viruses had been isolated from diarrhoeic calf faeces. The MRI isolates all originated from faecal samples which had been collected in Scotland between 1987 and 1989, and which were found to contain BCV on the basis of the results obtained in a diagnostic ELISA. At the start of this period, lamb anti-BCV (strains S1 and CK) serum (serum 5000) was used as the capture Ab in this assay. This polyclonal serum was later replaced by MAb S2/1, which may have influenced the BCV strains detected. The study could have been improved by including more MAbs and by examining isolates which had been obtained from a larger geographical area and collected over a longer period of time. Future work should aim at establishing whether BCVs associated with respiratory infections in calves and winter dysentery in adult cattle are identical to the calf enteric strains. Limited investigations with polyclonal sera and MAbs suggest that the calf enteric and respiratory strains are identical (Thomas et al, 1982; Vautherot and Laporte, 1983; McNulty et al, 1984; Reynolds et al, 1985). Reports comparing calf enteric isolates with winter dysentery isolates offer conflicting results (Akashi et al, 1980; Benfield and Saif, 1990).

The antigenic variations detected on the BCV isolates in these experiments also provide information on the epitope specificities of the MAbs. This has already been discussed in conjunction with the results obtained in epitope mapping studies in Chapter 5.

CHAPTER 7

ANIMAL MODELS OF BOVINE CORONAVIRUS INFECTION

CHAPTER 7
ANIMAL MODELS OF BOVINE CORONAVIRUS INFECTION

Introduction

The ability to reproduce infectious diseases in animals kept under experimental conditions facilitates study of the disease and permits challenge experiments to be performed in which protective antigens can be identified and different strains of the agent examined for their abilities to cross protect. Animal models of infection are also essential for vaccine development.

Gnotobiotic, SPF and colostrum-deprived conventional calves have been widely used as models of BCV infection. Following oral or intranasal inoculation, calves develop diarrhoea 1-5 days later. Diarrhoea lasts for 2 to 5 days, and is accompanied by virus excretion in the faeces. Virus is also excreted from the upper respiratory tract, but excretion is only occasionally accompanied by mild clinical signs (Mebus et al, 1975; Bridger et al, 1978b; Crouch et al, 1984; McNulty et al, 1984; Reynolds et al, 1985; Singh et al, 1985; Saif et al, 1986; Saif, 1987; Heckert et al, 1989; Kapil et al, 1990).

Attempts have been made to establish a small animal model of BCV infection, which would enable experiments to be performed on larger numbers of animals at a greatly reduced cost. In the ideal small animal model, oral inoculation with BCV would result in diarrhoea, accompanied by virus excretion in the faeces. El-Ghorr (1988) investigated the possibility of reproducing diarrhoea in gnotobiotic lambs and neonatal mice by oral inoculation with BCV. Gnotobiotic lambs proved unsuitable as models of BCV infection, as

only 1 out of 5 lambs developed diarrhoea after inoculation with the virus. Neonatal mice have been used as a model system for bovine rotavirus infections (Ramig, 1988), but attempts made by El-Ghorr (1988) to produce diarrhoea by oral inoculation with BCV were inconclusive. Fifteen percent of mice (12 out of 79 mice) developed diarrhoea after inoculation with cell culture harvest virus. This proportion increased to 70% (7 out of 10 mice) following inoculation with concentrated virus, but 50% of control mice (5 out of 10) inoculated with concentrated MI HRT-18 cell harvests also developed diarrhoea.

In common with many CVs (Wege et al, 1982), BCV has been successfully adapted to grow in the brains of suckling mice following intracerebral inoculation (Kaye et al, 1975; Bengelsdorff, 1988). The mice generally develop symptoms of encephalitis followed by death 4-5 days pi (Akashi et al, 1981; Gerna et al, 1981). Barthold et al (1990) demonstrated that intranasal inoculation of 1 day old mice could also cause encephalitis. No lesions were detected in either the respiratory or enteric tracts after intranasal inoculation. BCV at the third passage level in mouse brain caused death in 1 day old mice, rats and hamsters when inoculated by the intracerebral or subcutaneous routes (Akashi et al, 1981). These model systems are of very limited use for studying BCV infections, as they bear no resemblance to the disease which occurs under natural conditions. Bengelsdorff (1988) did however demonstrate that female mice vaccinated against BCV successfully transferred maternal immunity to their offspring, rendering them immune to challenge with the adapted BCV strains.

Further trials were performed to assess the possibility of using neonatal mice as experimental models of BCV enteric infection, and attempts were also made to infect a gnotobiotic piglet. A gnotobiotic calf was then inoculated with BCV by the oral and intransal routes. Virus excretion from the respiratory and enteric tracts was monitored, and the serological response studied.

Results

Neonatal mice

The possibility of using neonatal mice as models of BCV enteric infection was investigated. Litters of neonatal mice derived from stocks supplied to the MRI as MHV-free were raised under conventional conditions. Each mouse was inoculated with 0.1 ml of virus or control (MI HRT-18 cell harvest) preparation into the upper oesophagus, and monitored daily for up to 6 days for the presence of diarrhoea. When diarrhoea was present, gentle abdominal pressure resulted in the excretion of semi-fluid, yellow faeces. Unaffected mice either failed to excrete any faeces or excreted firm yellow or brown faeces when abdominal pressure was applied. Mice which died within 2 days of inoculation were excluded from the results. The percentage of mice which developed diarrhoea for at least 1 day of the trial was calculated, and the results obtained from non-infected mice and mice inoculated with virus or the control preparation compared.

In preliminary trials, 6 out of 26 mice (23%) developed diarrhoea after inoculation with PQ virus harvested from cell culture (IT = 5.3, HA titre = 80), whilst 13 out of 18 mice (72%)

developed diarrhoea after inoculation with concentrated PQ virus (IT = 7.8, HA titre = 400). S2 strain BCV was used in subsequent experiments because it could be grown to a higher titre than PQ virus. It was grown on HRT-18 cells, and concentrated by ultracentrifugation prior to inoculation.

Two trials (A and B) were conducted, and in each the baby mice were split into 3 groups of litters. One group did not receive any inoculum. The mice in a second group were each given 0.1 ml of concentrated MI HRT-18 cells (IT = <2.3, HA titre = <10), whilst those in the third group were each given 0.1 ml of concentrated S2 virus (in trial A, IT = 7.8, HA titre = 25600; in trial B, IT = 8.8, HA titre = 163,840). The mice were monitored daily for 4 days for the presence of diarrhoea. In trial A the mice were monitored by the candidate, whilst in trial B the mice were monitored by Dr. F. Scott of the MRI who had had considerable experience in monitoring mice infected with bovine rotavirus.

The results of trials A and B are summarised in Table 7.1. Statistical analysis was performed by Dr. Frank Wright of the Scottish Agricultural Statistics Service. 71% of the mice in trial A and 51% in trial B developed diarrhoea after inoculation with S2 virus. A high proportion of mice inoculated with MI HRT-18 cells also developed diarrhoea: 21% in trial A and 47% in trial B. Three mice in trial A which had not received any inoculum also developed diarrhoea. The results obtained in the 2 trials were pooled and analysed. Inoculation of mice with either MI HRT-18 cells or with S2 strain BCV had a significant effect on the number of mice which developed diarrhoea compared to the untreated

Table 7.1

Incidence of diarrhoea in mice inoculated with BCV.

Incidence of diarrhoea in mice inoculated with BCV

Inoculum	Number of mice with diarrhoea (%)		
	Trial A	Trial B	Trial A + B
None	3 / 20 (15%)	0 / 45 (0%)	3 / 65 (5%)
MI HRT-18 cells	6 / 29 (21%)	21 / 45 (47%)	27 / 74 (36%)
S2 strain BCV	20 / 28 (71%)	23 / 45 (51%)	43 / 73 (59%)

controls (probability (P) < 0.00001 for either group, using 2-tailed Fishers exact tests). A significantly greater proportion of mice developed diarrhoea after inoculation with S2 strain BCV than after inoculation with MI HRT-18 cells (P = 0.0052, using a 1-tailed Fishers exact test).

The results obtained in trials A and B were also pooled in order to compare the times of onset and the duration of diarrhoea between mice of the 3 groups (Figs. 7.1 and 7.2). All mice which developed diarrhoea did so within 4 days of inoculation, with the majority developing diarrhoea on the first or second day. Diarrhoea generally lasted for only one or sometimes two days, regardless of the inoculum given. The figures obtained for the onset of diarrhoea were analysed using a Generalised Linear Model, with binomial variance and a logit link. Differences between the 3 groups were tested by checking the statistical significance of the onset group interaction term in the model. This term was not found to be significant ($F_{2,8} = 0.65$; $F_{2,8}$ P = 0.05 (tables) = 19.37). A stastical analysis of the duration of diarrhoea was inappropriate, as only small numbers of mice had diarrhoea for more than one day. Study of these histograms (Fig. 7.2) however suggests that mice inoculated with BCV developed diarrhoea which lasted for a similar duration as mice inoculated with MI HRT-18 cells.

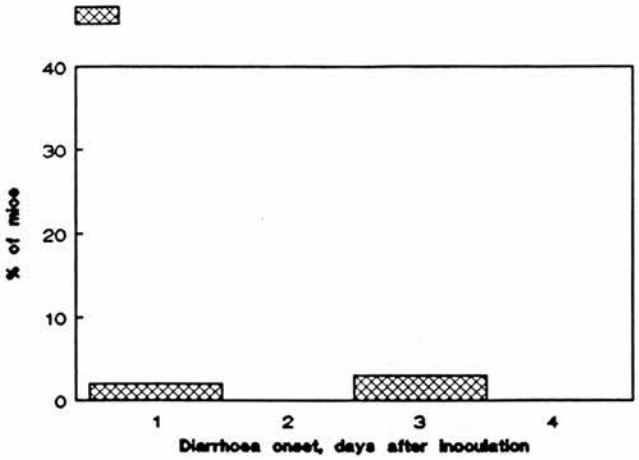
In 2 further trials attempts were made to detect BCV in the intestines harvested from mice with diarrhoea. Mice were inoculated orally with BCV or MI HRT-18 cells, and any mice which developed diarrhoea were immediately culled. The small and large

Figure 7.1

The onset of diarrhoea, days after inoculation, in mice either given no inoculum or inoculated with MI HRT-18 cells or S2 strain BCV. (Figures obtained from trials A and B: see text).

The % of inoculated mice in each group which developed diarrhoea 1, 2, 3 or 4 days after inoculation is indicated by the heights of the cross-hatched areas.

No inoculum



Inoculum = MI HRT-18 cells

Inoculum = S2 strain BCV

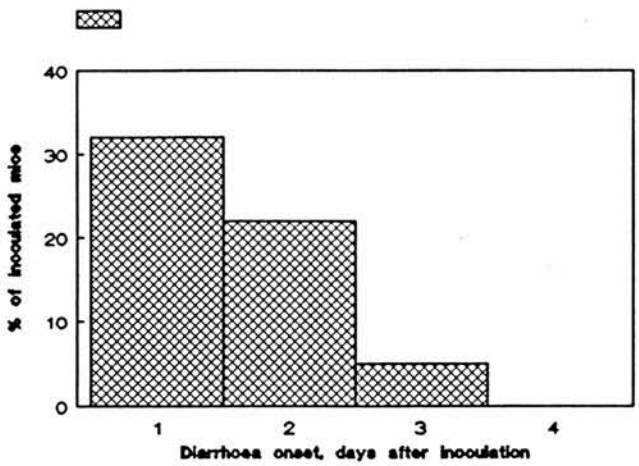
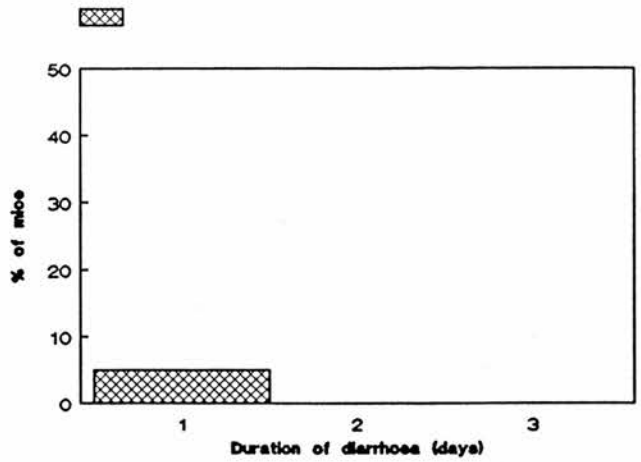


Figure 7.2

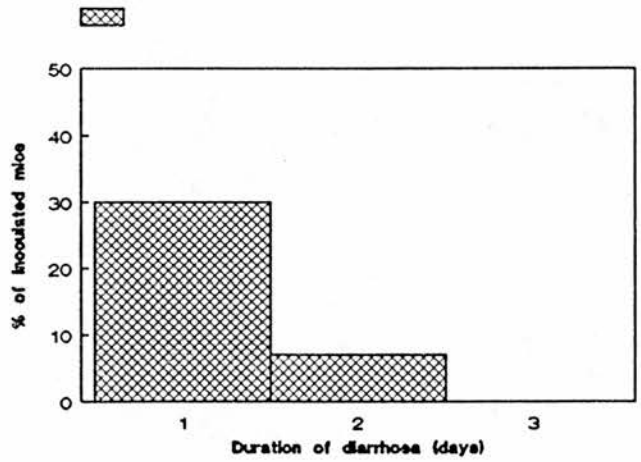
The duration of diarrhoea in mice either given no inoculum or inoculated with MI HRT-18 cells or S2 strain BCV (Figures obtained from trials A and B: see text).

The % of inoculated mice in each group which developed diarrhoea persisting for 1, 2 or 3 days is indicated by the heights of the cross-hatched areas.

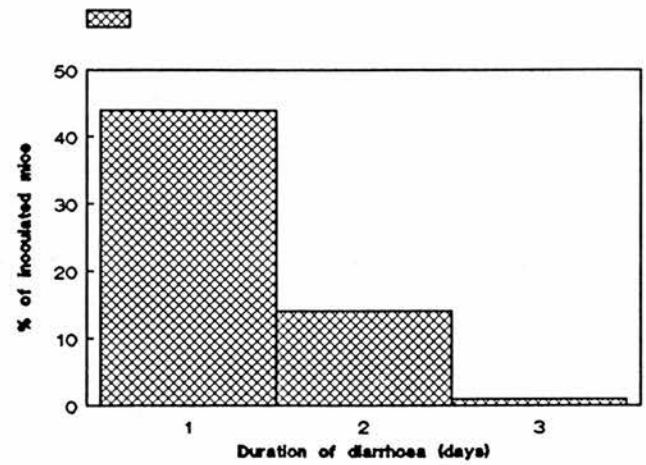
No inoculum



Inoculum = MI HRT-18 cells



Inoculum = S2 strain BCV



intestines were harvested and homogenised with appropriate volumes of RPMI MM to dilute them 1:10. The homogenates were clarified and the supernates tested for the presence of BCV by measurement of the ITs on HRT-18 cells and by measurement of the HA titres. Guts were harvested from 18 diarrhoeic mice inoculated with BCV and 15 diarrhoeic mice inoculated with MI HRT-18 cells. When the gut preparations were inoculated onto HRT-18 cells, IF staining failed to detect BCV Ags in any of the samples (IT <2.3). Non-specific agglutination occurred when the gut preparations were titrated in HA tests (HA titres = 10-80). The non-specific agglutinins were successfully extracted with the organic solvent Arklone (HA titres = <10), but no HA activity remained in the preparations from mice which had received BCV. Control virus samples demonstrated that Arklone treatment did not affect the ability of BCV to haemagglutinate. It thus proved impossible to detect BCV by measurement of either infectivity or HA titres in any of the gut preparations tested.

Gnotobiotic piglet

A 10 day old, male, colostrum-deprived gnotobiotic piglet was inoculated with a suspension of faeces containing S1 strain BCV. This virus had originated from a field case of BCV enteritis. It had been passaged by Dr. D. Snodgrass and Dr. A. El-Ghorr through 2 gnotobiotic calves (calves F690 and L965) and the faeces from calf L965 used to inoculate the piglet. The inoculum was prepared by making a 1:5 dilution of the faeces in PBS, homogenising and then spinning the suspension at 1000 rpm for 5 min on a bench

centrifuge. Filtration was avoided, in order to maintain the maximum possible dose of virus. Ten ml of supernatant fluid were inoculated orally and a further 1.0 ml inoculated into each nostril (day 0). The piglet was monitored daily for any clinical abnormalities and faecal and nasal swabs collected. Attempts were made to detect BCV in the faecal samples using the diagnostic ELISA and by IGEM (El-Ghorr et al, 1988), the latter technique being performed by Mrs. L. Inglis at MRI. Attempts were made to detect BCV antigens in the nasal swab samples by IF staining of acetone-fixed nasal epithelial cells using lamb anti-BCV serum (serum 5000) followed by pig anti-sheep Igs FITC conjugate. Serum samples were collected on days 4 and 12 and tested for BCV Abs in a SN test. The piglet was killed on day 12.

Inoculation of the piglet failed to produce any detectable signs of BCV infection or disease. The piglet remained bright throughout the experiment, and its temperature and appetite were normal. The appearance of the faeces was unchanged by inoculation and no virus was detected. A few samples, including the preinoculation sample, gave moderate OD readings (around 0.5) in the diagnostic ELISA. These were blocked if a 1:20 dilution of either lamb anti-BCV serum (serum 5000) or lamb anti-rotavirus serum (serum 3626) was added before the detecting Ab, suggesting that other components of the piglet faeces were binding in a non-specific manner in this test. IF staining of fixed nasal epithelial cells also failed to detect any BCV Ags. The serum samples collected on days 4 and 12 both had SN titres of <10.

Gnotobiotic calf

Method

A male, Friesian cross Limousin colostrum-deprived gnotobiotic calf was inoculated at 2 days old (day 0) with a preparation of the field faecal sample from which S2 virus had originally been isolated (El-Ghorr, 1988). This sample had been stored at -70^{0}C for several years, so it was first tested in the diagnostic ELISA to confirm that BCV was still present. Absence of rotavirus and other contaminating viruses was demonstrated by PAGE followed by silver staining (Herring et al, 1982), and by EM. A 10% suspension of a bacteria-free filtrate was prepared, and 19ml inoculated orally and a futher 5 ml inoculated into each nostril of the calf. It was inoculated IM on days 25 and 35 with 2.5 ml and 3.0 ml respectively of cell culture adapted S2 virus emulsified with FIA in a ratio of 1:2. The experiment was terminated on day 50, when the calf was removed from the isolator and a large volume of serum collected for use in routine laboratory tests.

The calf was monitored clinically each day, and its demeanour, rectal temperature and appetite (volume of milk drunk) recorded. During the first 11 days of the experiment, a bag was fitted to the rear of the calf by means of a harness. This allowed the faeces to be collected each day and their total mass and % dry matter (DM) were measured. During the latter course of the experiment faecal samples were collected with a swab. The faecal samples were tested for BCV using the diagnostic ELISA and by virus isolation in TOC. Nasal swab samples were collected by gently rubbing a 15 cm swab as far as possible up both nostrils. The

swabs were placed in 1.5 ml virus transport medium and mixed on a vortex mixer. Virus was detected by isolation in TOC or by IF staining of acetone-fixed nasal epithelial cells using either MAb S2/1 or rabbit anti-S2 serum (serum 5317) followed by the appropriate anti-species Ig conjugated with FITC. Serum samples were collected from the calf twice a week and titrated against S2 virus in IF, SN and HAI tests. The serum samples were also used to probe S2 virus proteins in Western blotting experiments and tested against the S2 MAbs in epitope blocking assays (EBAs).

Results relating to the alimentary and respiratory tracts

The observations and results relating to the alimentary tract are summarised in Table 7.2. The calf was moderately dull on day 5 at the onset of diarrhoea, but remained bright at other times. The animal was never pyrexic. His milk consumption dropped on day 2 and did not return to normal until day 8: the calf was particularly reluctant to drink on days 4 and 5. Diarrhoea commenced on day 5 and continued for 4 days, as demonstrated by the marked drop in the % DM of the faeces. During this time the faeces were brown and very fluid, and occasionally contained strands of gut lining and specks of blood. The faeces remained soft for a further 3 days before returning to normal on day 13. The mass of faeces excreted per day remained approximately constant at around 50g, with the exception of day 7 when 235g were excreted. BCV was detected in the faeces from day 4 until day 9, and was then intermittently detected until day 15, after which virus excretion ceased.

Table 7.2

Gnotobiotic calf infected with S2 strain BCV

Results relating to the alimentary tract

Days PI	Milk drunk* (ml)	Faeces			Detection of BCV in faeces	
		Texture**	Mass (g)	DM (%)	ELISA	isolation in TOC
0	5400	N	NT	NT	-	NT
1	6600	N	67	18	-	-
2	4400	N	51	23	-	-
3	4700	N	71	23	-	-
4	3900	N	45	18	+	+
5	3900	D	61	9	+	+
6	5400	D	73	6	+	+
7	5600	D	235	7	+	+
8	5900	D	85	9	+	+
9	6000	D	25	12	+	-
10	6000	(D)	40	14	-	-
11	6000	(D)	12	14	-	-
12	6000	(D)	NT	NT	+	-
13	6000	N	NT	NT	+	NT
14	6000	N	NT	NT	-	NT
15	6000	N	NT	NT	+	NT

*A maximum of 6 litres of milk were offered daily.
**N = normal D = diarrhoea (D) = slight diarrhoea
NT = not tested

The results relating to the respiratory tract are summarised in Table 7.3. The calf never showed any clinical signs relating to infection of the respiratory tract. IF staining of acetone-fixed nasal epithelial cells detected high levels of BCV Ags between days 1 and 8, with lower levels detected between days 9 and 12. MAb S2/1 proved to be a more sensitive probe and gave lower levels of background staining than rabbit anti-S2 serum (serum 5317). The binding of serum 5317 was blocked by first incubating the infected cells with lamb anti-BCV serum (serum 5000), demonstrating that the staining observed with serum 5317 was specific for BCV. IF was never observed when rabbit anti-rotavirus serum (serum 4381) was used as a negative control Ab. BCV was isolated in TOC from the nasal swab samples on days 2 to 8, corresponding to the period when large numbers of nasal epithelial cells were stained by IF.

Serological response to infection

The serum samples collected from the calf were titrated against S2 virus in IF, SN and HAI tests (Fig. 7.3). Ab was first detected by all 3 assays between days 7 and 11. IF titres continued to rise, being further boosted after vaccination on day 25. SN and HAI titres remained low until vaccination on day 25, when significant boosts occurred.

The serological responses of the calf to individual viral proteins and their antigenic regions were monitored by using the test sera as probes in Western blotting experiments and as competitor Abs in EBAs. In the Western blotting experiments, S2

Table 7.3

Gnotobiotic calf infected with S2 strain BCV

Detction of BCV in upper respiratory tract

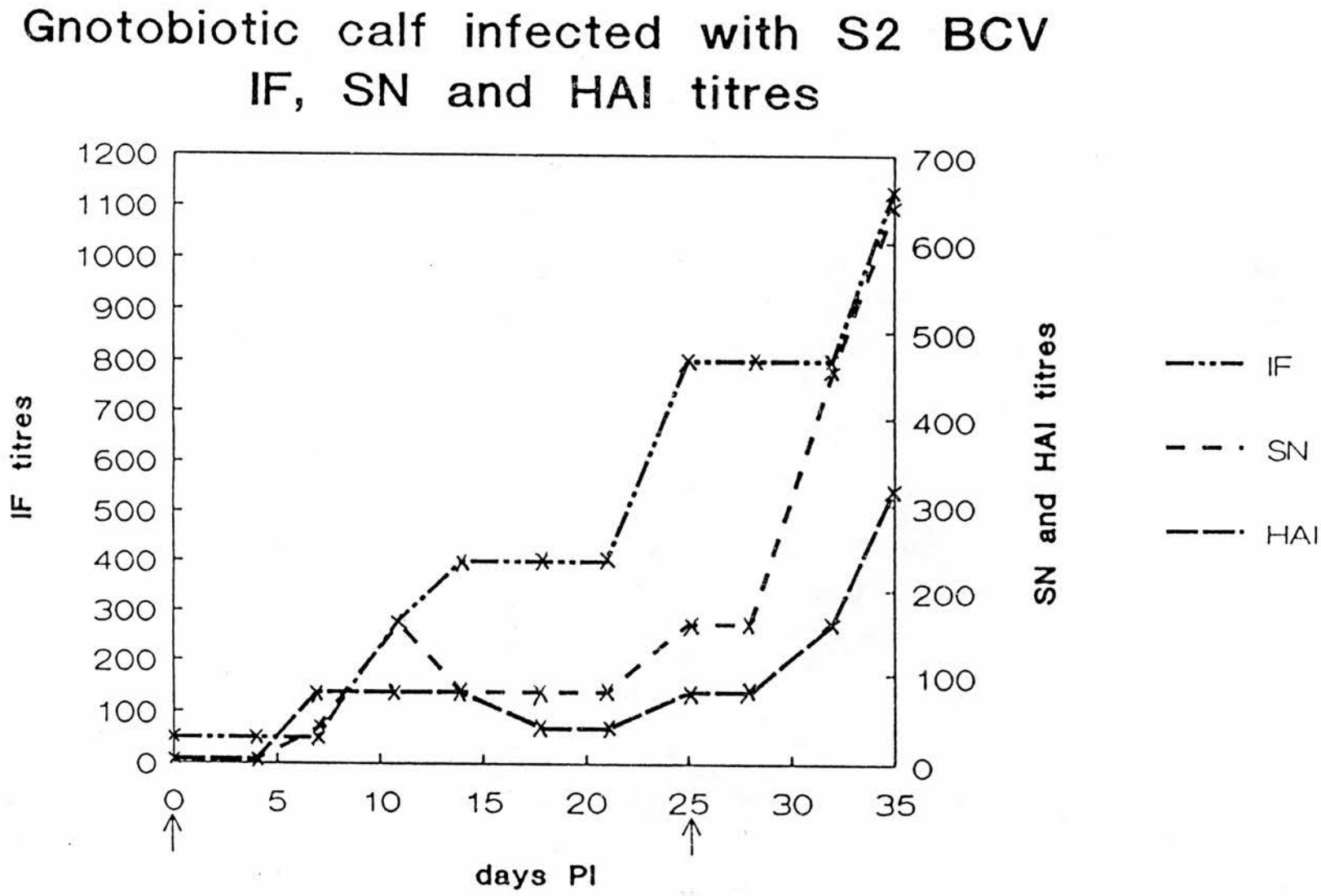
Days PI	Detection of BCV in nasal swab samples by :		
	IF * with :		Isolation in TOC
	MAb S2/1	Rabbit anti-S2 serum	
0	-	-	-
1	++	-	-
2	++	++	+
3	++	+	+
4	++	++	+
5	++	+	+
6	++	++	+
7	++	++	+
8	++	+	+
9	+	+	-
10	+	+	-
11	+	-	-
12	+	+	-
13	-	-	-
14	-	-	NT
15	-	-	NT

* The number of cells in which BCV antigens were detected by indirect IF were classified by eye as many (++), few (+) or none (-).

NT = not tested.

Figure 7.3

Gnotobiotic calf infected with S2 BCV: IF, SN and HAI titres.



The arrows indicate when the calf was inoculated with BCV by the oral/intranasal (day 0) and IM (day 25) routes.

virus proteins were dissociated by boiling with LSB in the absence of ME (non-reducing conditions), blotted onto nitrocellulose paper and probed with the consecutive serum samples (Fig. 7.4). Abs to the S, HE and N proteins were first detected at 11 days pi whilst Abs to the M gps were first detected very weakly at 21 days pi. Abs present in gnotobiotic lamb anti-MI HRT-18 cell sera (sera 5923 and 5926) also bound in these experiments to a cellular protein with a MW similar to that of the HE gp. This reaction did not interfere with the results obtained using the test sera, as Abs to the HE gp were detected before the calf received any cellular proteins when it was vaccinated on day 25.

The test sera were titrated against MAbs S2/1, S2/2, S2/3, S2/6 and S2/8 in EBAs. The mean OD values obtained with each test sample were plotted against the serum dilutions, and sigmoidal curves produced. End point ODs were defined so that they fell around the centre of the straight line regions of these curves, and so that the initial rises in Ab titres were detected. They were taken as 50% the mean $OD_{(no\ competitor)}$ value for the HE and S MAbs, and as 65% and 80% the mean $OD_{(no\ competitor)}$ value for the N MAbs S2/6 and S2/8 respectively. The mean $OD_{(no\ competitor)}$ values were obtained for each test plate from a set of wells to which PBS/0.05%T was added in place of competitor Abs. The reciprocals of the serum dilutions at the end point OD values were determined from the curves. These serum titres are shown in Table 7.4, and plotted against the days pi in Fig. 7.5.

Figure 7.4

Serological response of gnotobiotic calf to S2 virus infection, determined by Western blotting (non-reducing conditions)

The proteins of S2 strain BCV were separated by SDS/PAGE, blotted onto nitrocellulose paper and probed with consecutive serum samples from the calf. Bound Abs were detected with anti-species HRP conjugates.

Template

Lane No.		Probe
1	Calf samples	0
2		4
3	days pi:	7
4		11
5		14
6		18
7		21
8		25
9		28
10		32
11		35
12		50
13	Controls:)	
14	)	
15	)	Cow field serum samples
16	)	
17	Gnotobiotic lamb anti-MI HRT18 serum (serum 5923)	
18	Gnotobiotic lamb anti-MI HRT18 serum (serum 5926)	
19	Gnotobiotic lamb anti-rotavirus serum (serum 3626)	
20	PBS/0.5%T	
21	S2 MAb: S2/8 (N)	
22	CVL MAb: 5528 (M)	
23	CVL MAb: 5529 (S)	
24	S2 MAb: 5638 (HE)	

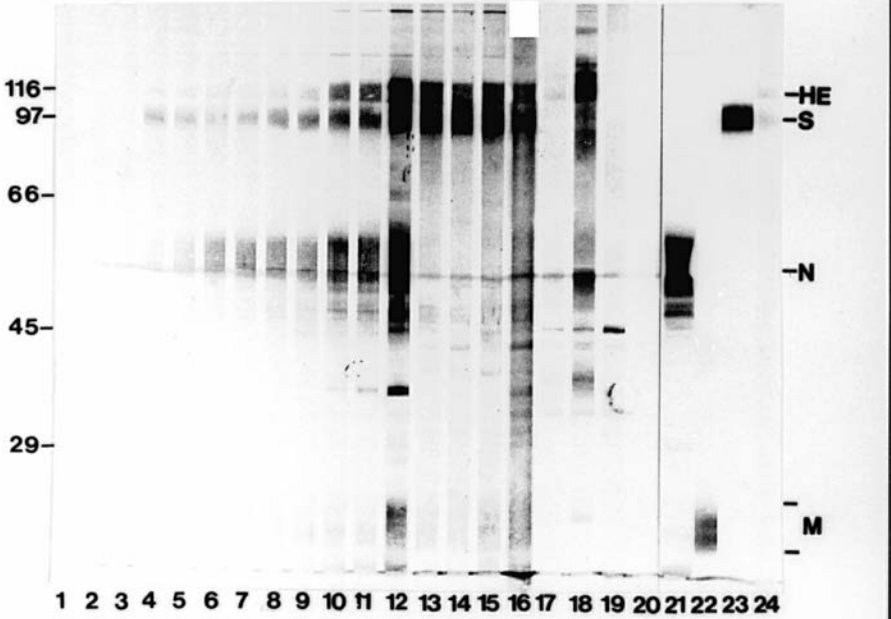

116
97
66
45
29
HE
S
N
M
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Table 7.4

Gnotobiotic calf infected with S2 strain BCV

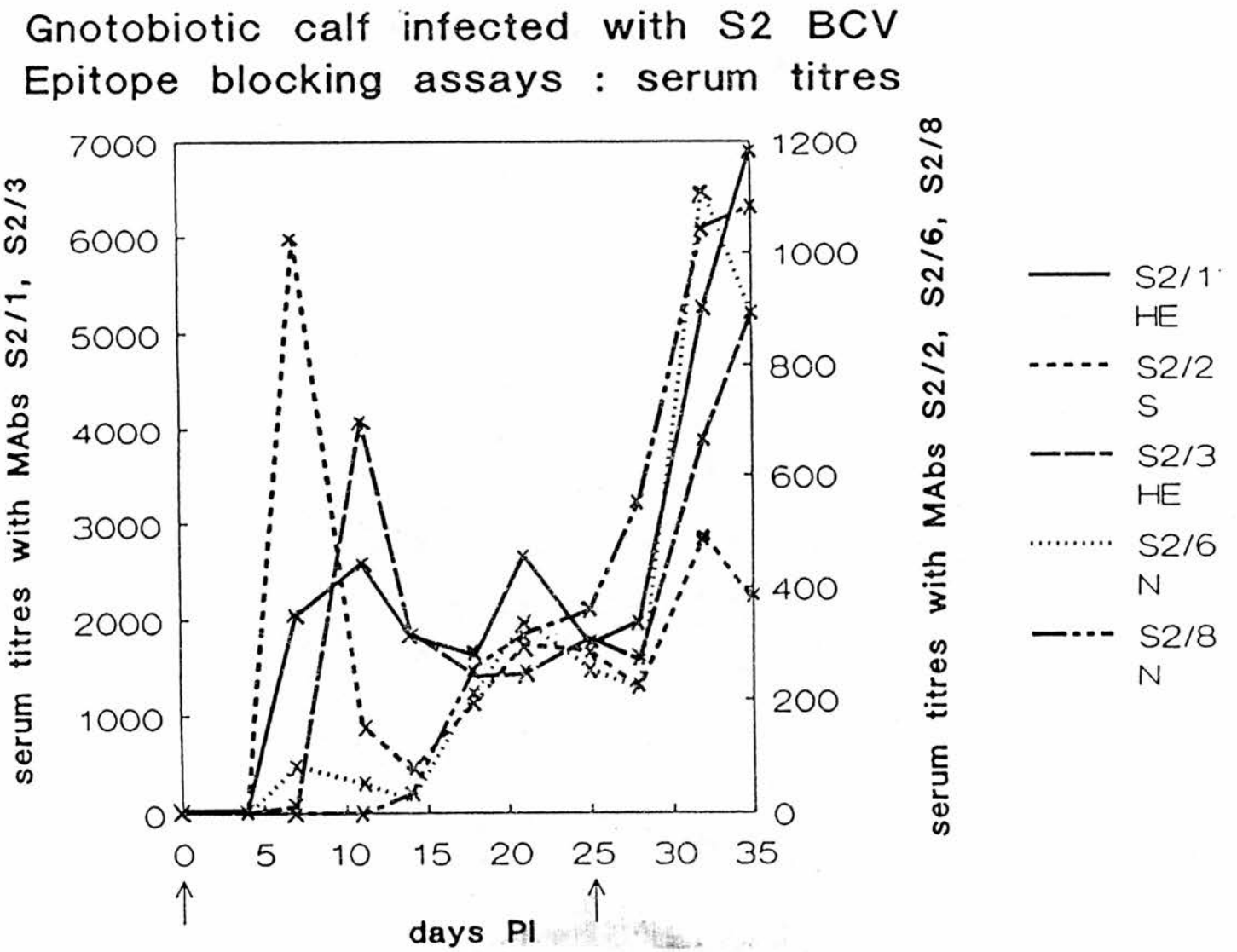
Results obtained in epitope blocking assays

Days PI	Serum titres obtained with MAbs* : S2/1 (HE)	S2/3 (HE)	S2/2 (S)	S2/6 (N)	S2/8 (N)
0	33	16	0	0	0
S2 virus inoculated orally and intranasally					
4	34	14	0	0	0
7	2042	74	1026	85	0
11	2587	4083	155	54	0
14	1838	1874	75	24	35
18	1632	1415	196	214	261
21	2671	1439	299	342	318
25	1752	1807	287	256	360
S2 virus inoculated IM					
28	1987	1584	221	219	554
32	5261	3918	492	1117	1046
35	6918	5195	376	893	1083
S2 virus inoculated IM					
50	84176	70431	6301	5013	1981

* Serum titres were defined as the reciprocals of the dilutions at the end point ODs. The end point ODs were defined as 50% the mean OD (no competitor) values for the HE and S MAbs and as 65% and 80% for the N MAbs S2/6 and S2/8 respectively.

Figure 7.5

Gnotobiotic calf infected with S2 BCV
Epitope blocking assays: Serum titres



The arrows indicate when the calf was inoculated with BCV by the oral/intranasal (day 0) and IM (day 25) routes.

The patterns of response obtained with EBAs using MAbs S2/1, S2/2, S2/3 and S2/6 were similar. The titres rose to peak at day 7 (MAbs S2/2 and S2/6) or day 11 (MAbs S2/1 and S2/3). They then fell to a minimum on day 14 (MAbs S/2 and S2/6) or day 18 (MAbs S2/1 and S2/3) before gradually rising again. Abs directed against the epitope delineated by MAb S2/8 were first detected on day 14 and rose gradually to day 25. Vaccination on days 25 and 35 significantly boosted the titres.

Discussion

Oral inoculation with BCV resulted in the production of diarrhoea in a significant proportion of neonatal mice, but the virus had no apparent effect on the time of onset or duration of diarrhoea. Neonatal mice must therefore be considered to be a potential model of BCV infection, but their use is limited by the very large numbers which would have to be studied in order to show significant differences between groups receiving S2 virus and MI HRT-18 cells. These results confirm the preliminary findings reported by El-Ghorr (1988). Mice which received MI HRT-18 cells probably developed diarrhoea due to the toxic effects of cell components or media constituents present in the inoculum. Any future experiments should include making a more rigorous effort to detect BCV in diarrhoeic mice, possibly by undertaking a time course study. The possibility of using other strains of BCV grown in alternative cell lines and of using different strains of mouse should also be investigated. Attempts could also be made to select a more virulent variant of the virus by repeated passage through mice.

The piglet tested in this experiment did not prove suitable as a model of BCV infection, and there is no documented evidence to suggest that BCV can infect swine. It may be significant that the CVs which infect pigs belong to antigenic group I, whereas BCV belongs to antigenic group II.

Diarrhoea was successfully reproduced in the calf, and was accompanied by virus excretion in the faeces. Virus continued to be detected intermittently in the faeces after diarrhoea had ceased, which supports the proposal that persistence of the virus in clinically normal animals may maintain a reservoir of BCV in infected herds (Heckert et al, 1990; Kapil et al, 1990). Infection of the upper respiratory tract was also demonstrated, but infection occurred in the absence of any clinical signs.

Consecutive serum samples were titrated in IF, SN and HAI tests. The IF titres reflect the total levels of BCV specific Abs present in the sera, whilst the SN tests measure the Abs directed against the neutralising epitopes on the S and HE gps. At present there is some controversy as to whether the HA activity of BCV resides on the HE and/or S gp (Storz et al, personal communication): the HAI titres are a measure of the Abs directed against the appropriate epitopes. The Western blotting experiment gave a qualitative indication of the Abs directed against each of the 4 structural proteins of BCV, whilst the EBAs measured the Ab responses to individual antigenic regions on these proteins. The former test was less sensitive, as Abs directed against the HE, S and N proteins were detected by the EBAs at a shorter interval pi. The results obtained in the Western blotting experiments demonstrated

that the serological response to the M gp occurred somewhat later than that to the other viral proteins.

When analysing the results of the EBAs, it is important to allow for the variations in the sensitivity and specificity of these tests. The sensitivities of the EBAs can be estimated by studying the results of the competition ELISAs described in Chapter 5. The concentration of homologous competitor MAb/well which gave levels of competition of 50% (MAbs S2/1, S2/2 and S2/3), 35% (MAb S2/6) and 20% (MAb S2/8) indicates the minimum amount of Ab which is necessary for a positive result in the EBAs. These can be calculated as 0.021 ug (MAb S2/1), 0.040 ug (MAb S2/2), 0.032 ug (MAb S2/3), 0.004 ug (MAb S2.6) and 0.237 ug (MAb S2/8). Assuming that MAbs and epitope specific Abs present in polyclonal sera have similar binding affinities, the EBAs based on MAbs S2/1, S2/2 and S2/3 are of similar sensitivities, whilst that based on MAb S2/6 is more sensitive and that based on MAb S2/8 is rather less sensitive. The end point OD for the EBA incorporating MAb S2/8 was defined as 80% the mean $OD_{(no\ competitor)}$ value, in an attempt to maximise the sensitivity of this test.

The specificities of the EBAs can also be established from the results obtained in the competition ELISAs (Chapter 5). The 4 HE MAbs all competed strongly with each other, even though other tests demonstrated that they were directed against 3 different epitopes. The HE MAbs also competed strongly with the S MAb (S2/2), so the EBAs incorporating the HE and S MAbs were unable to differentiate between Abs directed against epitopes delineated by these MAbs. The curves obtained using MAbs S2/1, S2/3 and S2/2 in EBAs were

therefore similar to each other. They were also similar to those obtained in the SN and HAI tests, which is expected as the HE and S gps carry the epitopes involved in neutralisation and haemagglutination. The very high peak which was detected on day 7 by the S2/2 EBA is difficult to explain, but may have been caused by other components present in the serum sample competing in a non-specific manner with this MAb.

MAbs S2/6 and S2/8 were used in EBAs to measure the serological responses directed against the N protein. The results obtained using these 2 MAbs were very different: this was not unexpected as competition ELISAs demonstrated that these MAbs were directed against different epitopes (Chapter 5). When MAb S2/6 was used as a detecting Ab in competition ELISAs, moderate levels of competition occurred with the HE MAbs. The end point of the S2/6 EBA was therefore defined as 65% the mean OD (no competitor) value, so that very high levels of HE Abs would have to be present before they had a significant effect on the titre measured by this EBA. The EBAs showed that the serological response directed against the epitope delineated by MAb S2/8 was slow and weak compared to that generated against other epitopes, but this may have been an artifact caused by the relatively low sensitivity of the S2/8 EBA.

Although only a single calf was used in these experiments, the results obtained confirm that colostrum-deprived, gnotobiotic calves are suitable as experimental models of BCV infection. Other reports suggest that conventionally derived, SPF calves may also be suitable as models of BCV infection, provided they are deprived of colostrum (Saif et al, 1986; Saif, 1987). El-Ghorr (1988)

failed to reproduce clinical disease in colostrum fed SPF calves, although the virus could occasionally be detected in the faeces.

CHAPTER 8

GENERAL DISCUSSION

CHAPTER 8
GENERAL DISCUSSION

Introduction

The main aim of this study was to establish the extent of antigenic variation amongst BCV isolates. Efficient techniques were first developed for isolating BCVs from faecal samples, and a panel of MAbs were raised against S2 strain BCV and characterised. This allowed a comprehensive investigation of BCV strain variation to be undertaken. The structural proteins of BCV were also analysed on a functional basis and the epitopes of the N and HE proteins partially mapped. Neonatal mice and a gnotobiotic piglet did not prove suitable as experimental models of BCV enteric infection, but disease was successfully reproduced in a colostrum deprived, gnotobiotic calf. In this Chapter, these different topics are discussed in more detail, and areas worthy of future research outlined.

Detection of BCV in calf faecal samples

Faecal samples collected from diarrhoeic calves were submitted to the MRI for testing for BCV using a diagnostic ELISA. Positive samples were used as the source of the BCV isolates which were subsequently examined for the presence of strain variations. At the start of these studies, the ELISA was based on polyclonal capture and detecting Abs, but the capture Ab was later replaced by MAb S2/1. There was therefore a danger that this MAb biased the panel of BCV isolates used in subsequent investigations. In the future, the diagnostic ELISA could be improved by replacing MAb

S2/1 with a pool of 2 or more MAbs directed against epitopes conserved between different BCV isolates (Czerny and Eichhorn, 1989). Ideally these MAbs should be directed against both internal and external virus proteins so that both whole and degraded virus particles are detected (Crouch et al, 1984). A mixture of MAbs S2/6 (N) and S2/7 (HE) would satisfy these requirements. To ensure new BCV strains are not missed during diagnosis, it might be worthwhile periodically examining a proportion of the faecal samples found negative on the ELISA by EM of negatively stained preparations.

Isolation of BCV

Tracheal organ cultures obtained from neonatal and young calves proved highly successful for the primary isolation of BCVs from faecal samples, and the viruses could then be adapted to growth in HRT-18 cells. During isolation and adaptation, it was important to monitor virus growth by a test which would not discriminate between different strains of the virus. Measurement of HA titres using rat RBCs was therefore chosen as the initial screening test, as all isolates of BCV tested to date have been found to agglutinate RBCs from this species. The only exceptions are some isolates originating from cows with winter dysentery, which cannot agglutinate either mouse or rat RBCs (Benfield and Saif, 1990). Samples giving positive results in HA tests were confirmed to be BCV specific by testing TOC samples in the diagnostic ELISA, and by using anti-BCV sera to stain infected HRT-18 cells in IF tests.

Viruses adapted to growth in HRT-18 cells were all grown under the conditions previously found in this laboratory to be optimal for the growth of M strain BCV (El-Ghorr, 1988). A more rigorous approach would probably have resulted in the growth of these viruses to higher titres, but this was not considered necessary for these studies.

Monoclonal antibodies against BCV

The unique specificities of MAbs make them ideal tools for analysing virus proteins. Studies using MAbs are always however limited by the number of MAbs which are available, and it is therefore important to obtain as large and as diverse a panel of MAbs as possible. Four MAbs directed against CVL strain BCV and a further 8 MAbs directed against S2 strain BCV were used in these studies. Of the 8 MAbs raised against S2 virus, 4 were directed against the HE gp, 1 against the S gp and 3 against the N protein. The high proportion of HE and N MAbs suggests either that the original preparations used to immunise the donor mice during MAb production were rich in these proteins, or that the mice responded more strongly to these antigens.

Characterisation of the structural proteins of S2 strain BCV

MWs

The MWs of the S2 virus proteins were determined by Western blotting using both polyclonal sera and MAbs as probes. The values of 116 (reducible to 64), 98, 52 and 21 (range 19 to 23) KD for

the HE, S, N and M proteins respectively agreed closely with published values for other isolates of the virus (King and Brian, 1982; Deregt et al, 1983).

Functional characterisation using MAbs

The 8 S2 MAbs and the 4 CVL MAbs were tested against S2 strain BCV in IF, SN and HAI tests. This served to characterise the MAbs, and also helped define the virus proteins important in neutralisation and haemagglutination. The results obtained with the CVL MAbs should be interpreted with caution, as their failure to react in SN or HAI tests may have been due to lack of epitope conservation on S2 virus.

The MAbs directed against the M and N proteins reacted with S2 strain BCV in IF tests only. Neutralising epitopes have so far never been detected on these proteins (Deregt and Babiuk, 1987), but MAbs directed against the M and N proteins of other CVs can both neutralise in vitro and protect in vivo (Buchmeier et al, 1984; Lecomte et al, 1987). It is therefore possible that a larger panel of MAbs might also detect neutralising epitopes on the M and N proteins of BCV. The results obtained in SN tests with the MAbs directed against the S and HE gps of S2 strain BCV clearly demonstrated that these proteins bear the major neutralising epitopes. This supports the findings of other groups (Vautherot and Laporte, 1983; Deregt and Babiuk, 1987). Future work should establish which epitopes are important for protection in vivo, as MAbs which neutralise the virus in vitro do not necessarily also protect in vivo (Deregt et al, 1989a).

The 4 S2 MAbs directed against the HE gp all inhibited virus induced haemagglutination of rat RBCs. The S2 MAb directed against the S gp was also a weak inhibitor of virus induced haemagglutination. Other groups have also reported that some S MAbs cause HAI (Vautherot and Laporte, 1983; Dea and Tijssen, 1989a; Storz et al, personal communication). Since MAbs directed against both the S and HE gps can cause HAI, it is possible that haemagglutination is caused by the combined effect of both these gps or by the individual effect of either one of these proteins. The ability of the HE gp to cause haemagglutination has recently been questioned, as whilst whole BCV particles agglutinated chicken RBCs, isolated HE gps failed to cause haemagglutination (Schultze et al, 1991). If the HE gp is not a haemagglutinin, HE MAbs which cause HAI must do so either by steric hindrance or by inducing conformational changes in the S gp (Storz et al, personal communication). These proposals are supported by results obtained by the candidate in competition ELISAs, in which HE MAbs inhibited binding of the S MAb, presumably because the HE MAbs exerted some effect on the S gp. An alternative explanation for the failure of isolated HE gps to cause haemagglutination is that these proteins need to be incorporated as part of a larger structure before they can agglutinate RBCs. Further work must be undertaken to establish whether the HE gp is directly involved with haemagglutination, and to prove that the S gp is indeed a viral haemagglutinin.

Epitope mapping of the N and HE proteins

The 3 S2 MAbs directed against the N protein each mapped to a

distinct antigenic region in competition ELISAs. The S2/5 MAb could not be used as a biotinylated detecting MAb, but its unique reactions with the standard strains of BCV in IF tests confirmed that it was directed against an epitope distinct from those defined by MAbs S2/6 and S2/8. This is the first report of the presence of distinct antigenic regions on the N protein of BCV.

The 4 S2 MAbs directed against the HE gp all competed strongly with each other in competition ELISAs, so in these tests they defined a single antigenic region. This region could be subdivided into 3 epitopes on the basis of results obtained in other tests. MAb S2/1 mapped to a unique epitope, on the basis of its failure to bind to blotted viral proteins and its reactions with certain virus isolates in ELISAs, IF and HAI tests. MAbs S2/3 and S2/4 were differentiated from MAbs S2/1 and S2/7 by their failure to neutralise CK and M strains of BCV. One antigenic region was therefore defined by MAb S2/1, a second by MAbs S2/3 and S2/4 and a third by MAb S2/7.

A larger panel of MAbs would allow a more complete definition of the antigenic regions and epitopes of the structural proteins of BCV. Identification of the epitopes important for <u>in vivo</u> protection is particularly important, as this is relevant for future vaccine development and facilitates accurate predictions concerning the effect of antigenic variations at particular sites. So far the antigenic regions of the BCV proteins have largely been defined on a functional basis. A structural definition may be achieved by analysis of degraded virus proteins, by generating and

sequencing neutralisation resistant mutants or by studying synthetic peptides.

Strain comparison of BCV isolates

Knowledge of the extent of strain variation is important when considering methods of controlling BCV diarrhoea and during vaccine development. Diagnostic tests should be based on the detection of viral epitopes which are known to be conserved between different strains of the virus. Previous studies of BCV strain variation had been limited to examining a relatively small number of isolates. These studies had demonstrated only minor variations in physicochemical properties (Dea et al, 1980b) and in reactions with polyclonal sera and MAbs (Dea et al, 1982; Vautherot and Laporte, 1983; El-Ghorr et al, 1989).

In the studies performed by the candidate, 5 standard strains of BCV (S1, S2, CK, M and PQ) were first examined for strain variations using polyclonal sera and MAbs. The study was then widened by using the S2 MAbs to screen 28 TOC isolates for strain variations, and from these results 7 viruses were chosen for adaptation to growth in HRT-18 cells (MRI BCV isolates). In these studies, strain variations were detected with the S2 MAbs on the N, HE and S proteins. The M gps could not be analysed with MAbs, as an insufficient amount of this MAb was available. Variations were most commonly detected on the HE and S gps, and these were most readily detected in SN tests. This finding was not unexpected, as greater selection pressure is exerted on epitopes involved in neutralisation. Other workers have also detected antigenic

variations on the HE and S gps of BCV with MAbs (Vautherot and Laporte, 1983; Deregt et al, 1989a; El-Ghorr et al, 1989). Strain variations were detected on the N protein with MAb S2/5 in IF tests: this is the first report of strain variation on the N protein of BCV. The other 2 N MAbs, S2/6 and S2/8, failed to detect antigenic variations on any of the isolates tested, and these MAbs would therefore be highly suitable for use in diagnostic tests.

Strain variations were never detected in these studies with polyclonal sera, which suggests that all the isolates belong to a single serotype. The MAbs detected wide variations in SN tests, whilst cross SN tests with rabbit polyclonal sera failed to detect antigenic variations on the 5 standard strains of BCV, and rabbit anti-S2 serum failed to detect variations amongst the 7 MRI isolates in SN tests. Shortage of time unfortunately precluded raising polyclonal sera against the 7 MRI BCV isolates and using these sera in cross SN tests. These findings emphasise the importance of using both polyclonal sera and MAbs in studies of antigenic variation. Whilst MAbs are excellent at discriminating between different virus isolates, there is always a danger that a biased panel of MAbs may emphasise antigenic similarities or minor differences that are of no relevance in vivo.

This study is the largest investigation of BCV strain variation which has been undertaken to date, and is important because it clearly demonstrates that antigenic variations do occur. Whilst the in vivo significance of these variations is unclear at present, their very existence suggests there is the potential for

significant variations to arise in the future. In any investigation of strain variation, it is important to study as wide a variety of isolates as possible. In this study, all the TOC isolates derived from faecal samples which had been collected in Scotland between 1987 and 1989. As discussed earlier, the diagnostic test for BCV and the virus isolation procedure may have led to some degree of selection of the isolates. Future studies could utilise larger and more diverse panels of viruses, MAbs and polyclonal sera. The data obtained in serological tests should also be compared with that obtained using molecular techniques such as hybridisation assays or sequencing data.

Animal models of BCV infection

Studies on BCV have been hampered by the lack of a small animal model of enteric BCV infection. The virus has been successfully adapted to grow in the brains of suckling mice following intracerebral or intranasal inoculation, but such a model is of little practical use (Kaye et al, 1975; Akashi et al, 1981; Barthold et al, 1990). In the ideal small animal model system, oral inoculation with BCV would result in diarrhoea, accompanied by virus excretion in the faeces and a serological response. Such a model would permit challenge experiments to be performed, in which protective antigens and epitopes could be identified and different strains analysed for their abilities to cause cross protection. A small animal model would also be useful during the early stages of vaccine development.

In these studies, neonatal mice and a gnotobiotic piglet did not prove suitable as animal models of BCV infection. The possibility of using other small laboratory animals such as rats or guinea pigs has not yet been investigated. El-Ghorr (1988) failed to consistently reproduce diarrhoea in gnotobiotic lambs orally infected with the virus. BCV diarrhoea was successfully reproduced in the colostrum deprived, gnotobiotic calf, which was orally and intranasally infected with S2 virus. BCV was excreted from both the respiratory and enteric tracts, and there was a marked serological response to infection. This study confirmed that colostrum deprived calves are the only suitable models of enteric BCV infection. In this study a gnotobiotic calf was used, but conventionally derived SPF calves may also be suitable (Saif et al, 1986).

APPENDIX

APPENDIX
RECIPES FOR BUFFERS AND SOLUTIONS

10X PBS

Sodium chloride		800g
Potassium chloride		20g
Disodium hydrogen phosphate (Na_2HPO_4)		115g
Potassium dihydrogen phosphate (KH_2PO_4)		20g
DW	to	10 l

30% acrylamide solution

Acrylamide (BDH)		252.0g
N,N'-methylenebisacrylamide (BDH)		6.72g
DW	to	610 ml

The solution was filtered through Whatman filter paper number 1.

2X Laemmli stacking gel buffer

Tris (Boehringer Mannheim GmbH)	3.03g
Sodium dodecyl sulphate (SDS) (BDH)	0.20g
DW	100 ml

The pH was adjusted to 6.8 with concentrated hydrocholoric acid (HCl).

4X Laemmli gel buffer

Tris	18.16g
SDS	0.40g
DW	100 ml

The pH was adjusted to 8.8 with concentrated HCl.

4X Laemmli sample buffer

2X Laemmli stacking gel buffer	5.0g
Sucrose	4.0g
DW	2.5g
Bromophenol blue	8 mg

2X Laemmli sample buffer (No ME)

4X Laemmli sample buffer	1.0 ml
10% SDS	400 ul
DW	600 ul

2X Laemmli sample buffer (with ME)

4X Laemmli sample buffer	1.0 ml
10% SDS	400 ul
2-Mercaptoethanol (ME) (BDH)	80 ul
DW	520 ul

Cracking buffer

SDS	1.0g
ME	2.5ml
Glycerol	5.0ml
DW	2.5ml
Bromophenol blue	8mg

3% polyacrylamide stacking gel

30% Acrylamide solution	1.5ml
2X Laemmli stacking gel buffer	7.5ml
DW	6.0ml

The solution was degased under vacuum and polymerisation initiated by the addition of 15 ul N,N,N',N'-tetramethyl-ethylenediamine (Temed) and 75 ul 10% ammonium persulphate solution.

7.5% polyacrylamide resolving gel

30% Acrylamide solution	10.0ml
4X Laemmli gel buffer	10.0ml
DW	18.7ml
Sucrose	2.0g

The solution was degased under vacuum and polymerisation initiated by the addition of 30ul Temed and 300ul 10% ammonium persulphate solution.

10% Polyacrylamide resolving gel

30% Acrylamide solution	20.0ml
4X Laemmli gel buffer	15.0ml
DW	24.1ml
Sucrose	3.0g

The solution was degased under vacuum and polymerisation initiated by the addition of 30ul Temed and 150ul 10% ammonium persulphate solution.

Laemmli electrode buffer

Tris	15.1g
SDS	5.0g
Glycine	72.1g
DW	5000ml

The pH of the solution was measured to ensure that it was correct at pH 8.3.

Tris/Glycine electrode blotting buffer

Tris	12.0g
Glycine	58.0g
Methanol	1000ml
DW to	5000ml

The pH of the solution was measured to ensure that it was correct at pH 8.3.

RIPA buffer

Tris/HCl pH 7.0	3.0g
Sodium chloride	4.4g
Sodium deoxycholate	5.0g
Triton X-100	5.0ml
Phenylmethylsulphonylfluoride (PMSF)	0.09g
DW to	500ml

MW markers: Sigma MW-SDS-200 kit

Carbonic anhydrase (bovine erythrocytes)	29 KD
Albumin (egg)	45 KD
Albumin (bovine plasma)	66 KD

Phosphorylase B (rabbit muscle)		97 KD
B-galactosidase (Escherichia coli)		116 KD
Myosin (rabbit muscle)		205 KD

Coomassie Brilliant Blue Stain

Coomassie Brilliant Blue R (Sigma)		0.2g
100% Trichloroacetic acid		5.0ml
Glacial acetic acid		37.0ml
Methanol (BDH)		250ml
DW	to	500ml

Destaining solution for Coomassie Brilliant Blue stain

Glacial acetic acid		35ml
Absolute alcohol		115ml
DW	to	500ml

The destaining solution used prior to drying gels was supplemented with 3% glycerol.

REFERENCES

REFERENCES

Abraham, S., Kienzle, T.E., Lapps, W. and Brian, D.A. (1990a). Deduced sequence of the bovine coronavirus spike protein and identification of the internal proteolytic cleavage site. Virology, 176, 296-301.

Abraham, S., Kienzle, T.E., Lapps, W.E. and Brian, D.A. (1990b). Sequence and expression analysis of potential non-structural proteins of 4.9, 4.8, 12.7 and 9.5 KDa encoded between the spike and membrane protein genes of the bovine coronavirus. Virology, 177, 488-495.

Acres, S.D., Laing, C.J., Saunders, J.R. and Radostits, O.M. (1975). Acute undifferentiated neonatal diarrhea in beef calves. 1. Occurrence and distribution of infectious agents. Canadian Journal of Comparative Medicine, 39, 116-132.

Aguila, H.L., Pollock, R.R., Spira, G. and Scharff, M.D. (1986). The production of more useful monoclonal antibodies. 2. The use of somatic-cell genetic and recombinant-DNA technology to tailor-make monoclonal antibodies. Immunology Today, 7, 380-383.

Akashi, H., Inaba, Y., Miura, Y., Tokuhisa, S., Sato, K. and Satoda, K. (1980). Properties of a coronavirus isolated from a cow with epizootic diarrhea. Veterinary Microbiology, 5, 265-276.

Akashi, H., Inaba, Y., Miura, Y., Sato. K., Tokuhisa, S., Asagi, M. and Hayashi, Y. (1981). Propagation of the Kakegawa strain of bovine coronavirus in suckling mice, rats and hamsters. Archives of Virology, 67, 367-370.

Almeida, J.D., Berry, D.M., Cunningham, C.H., Hamre, D., Hofstad, M.S., Mallucci, L., McIntosh, K. and Tyrrell, D.A.J. (1968). Coronaviruses. Nature, London, 220, 650.

Archetti, I. and Horsfall, F.L. (1950). Persistent antigenic variation of influenza A viruses after incomplete neutralization in ovo with heterologous immune serum. Journal of Experimental Medicine, 92, 441-462.

Armstrong, J., Niemann, H., Smeekens, S., Rottier, P. and Warren, G. (1984). Sequence and topology of a model intracellular membrane protein, E1 glycoprotein, from a coronavirus. Nature, London, 308, 751-752.

Babiuk, L.A., Sabara, M. and Hudson, G.R. (1985). Rotavirus and coronavirus infections in animals. Progress in Veterinary Microbiology and Immunology, 1, 80-120.

Barthold, S.W., Souza, M.S. and Smith, A.L. (1990). Susceptibility of laboratory mice to intranasal and contact infection with coronaviruses of other species. Laboratory Animal Science, 40, 481-485.

Battaglia, M., Lutz, H. and Wyler, R. (1986). Serologische ubersichtsuntersuchung uber die verbreitung des bovinen Coronavirus in der Schweiz. Schweizer Archiv fur Tierheilkunde, 128, 213-218.

Beaudette, F.R. and Hudson, C.B. (1937). Cultivation of the virus of infectious bronchitis. Journal of the American Veterinary Medical Association, 90, 51-60.

Benfield, D.A. and Saif, L.J. (1990). Cell culture propagation of a coronavirus isolated from cows with winter dysentery. Journal of Clinical Microbiology, 28, 1454-1457.

Bengelsdorff, H.J. (1988). Vaccination of cows against coronavirus-infection in their offspring: The mouse as a laboratory animal model. Berliner und Munchener Tierarztliche Wochenschrift, 8, 278-282.

Bengelsdorff, H.J., Bernhardt, D., Pranter, W. and Wieda, J. (1989). Maternal vaccination against calf diarrhea pathogens-Efficacy trials in the mouse as an alternative model to the cow. Tierarztliche Umschau, 44, 358-364.

Berry, D.M., Cruickshank, J.G., Chu, H.P. and Wells, R.J.H. (1964). The structure of infectious bronchitis virus. Virology, 23, 403-407.

Birk, H.W. and Koepsell, H. (1987). Reaction of monoclonal antibodies with plasma membrane proteins after binding on nitrocellulose: Renaturation of antigenic sites and reduction of nonspecific antibody binding. Analytical Biochemistry, 164, 12-22.

Bjorck, L. and Kronvall, G. (1984). Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. Journal of Immunology, 133, 969-974.

Boireau, P., Cruciere, C. and Laporte, J. (1990). Nucleotide sequence of the glycoprotein S gene of bovine enteric coronavirus and comparison with the S proteins of two mouse hepatitis virus strains. Journal of General Virology, 71, 487-492.

Boyle, J.F., Weismiller, D.G. and Holmes, K.V. (1987). Genetic resistance to mouse hepatitis virus correlates with absence of virus-binding activity on target tissues. Journal of Virology, 61, 185-189.

Brian, D.A., Hogue, B., Lapps, W., Potts, B. and Kapke, P. (1983). Comparative structure of coronaviruses. In Proceedings, Fourth International Symposium on Neonatal Diarrhea, pp. 100-116. VIDO, Saskatchewan.

Bridger, J.C., Caul, E.O. and Egglestone, S.I. (1978a). Replication of an enteric bovine coronavirus in intestinal organ cultures. Archives of Virology, 57, 43-51.

Bridger, J.C., Woode, G.N. and Meyling, A. (1978b). Isolation of coronaviruses from neonatal calf diarrhoea in Great Britain and Denmark. Veterinary Microbiology, 3, 101-113.

Broes, A., Van Opdenbosch, E. and Willemans, G. (1984). Isolement d'un coronavirus chez des bovins atteints d'enterite hemorragique hivernale (winter dysentery) en Belgique. Annales de Medecine Veterinaire, 128, 299-303.

Buchmeier, M.J., Lewicki, H.A., Talbot, P.J. and Knobler, R.L. (1984). Murine hepatitis virus-4 (strain JHM)-induced neurologic disease is modulated in vivo by monoclonal antibody. Virology, 132, 261-270.

Bulgin, M.S., Ward, A.C.S., Barrett, D.P. and Lane, V.M. (1989). Detection of rotavirus and coronavirus shedding in two beef cow herds in Idaho. Canadian Veterinary Journal, 30, 235-239.

Burnette, W.N. (1981). "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Analytical Biochemistry, 112, 195-203.

Callebaut, P., Pensaert, M.B. and Hooyberghs, J. (1989). A competitive inhibition ELISA for the differentiation of serum antibodies from pigs infected with transmissible gastroenteritis virus (TGEV) or with the TGEV-related porcine respiratory coronavirus. Veterinary Microbiology, 20, 9-19.

Cavanagh, D. (1983). Coronavirus IBV: Structural characterization of the spike protein. Journal of General Virology, 64, 2577-2583.

Cavanagh, D. and Davis, P.J. (1986). Coronavirus IBV: Removal of spike glycopolypeptide S1 by urea abolishes infectivity and haemagglutination but not attachment to cells. Journal of General Virology, 67, 1443-1448.

Cavanagh, D., Brian, D.A., Enjuanes, L., Holmes, K.V., Lai, M.M.C., Laude, H., Siddell, S.G., Spaan, W., Taguchi, F. and Talbot, P.J. (1990). Recommendations of the coronavirus study group for the nomenclature of the structural proteins, mRNAs, and genes of coronaviruses. Virology, 176, 306-307.

Cepica, A., Yason, C. and Ralling, G. (1990). The use of ELISA for detection of the antibody-induced conformational change in a viral protein and its intermolecular spread. Journal of Virological Methods, 28, 1-14.

Cheever, F.S., Daniels, J.B., Pappenheimer, A.M. and Bailey, O.T. (1949). A murine virus (JHM) causing disseminated encephalomyelitis with extensive destruction of myelin. I. Isolation and biological properties of the virus. Journal of Experimental Medicine, 90, 181-194.

Collins, A.R., Knobler, R.L., Powell, H. and Buchmeier, M.J. (1982). Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. Virology, 119, 358-371.

Collins, J.K., Riegel, C.A., Olson, J.D. and Fountain, A. (1987). Shedding of enteric coronavirus in adult cattle. American Journal of Veterinary Research, 48, 361-365.

Cox, G.J., Parker, M.D. and Babiuk, L.A. (1989). The sequence of cDNA of bovine coronavirus 32K nonstructural gene. Nucleic Acids Research, 17, 5847.

Crouch, C.F. and Raybould, T.J.G. (1983). Comparison of different antigen preparations as substrates for use in passive hemagglutination and enzyme-linked immunosorbent assays for detection of antibody against bovine enteric coronavirus. Journal of Clinical Microbiology, 18, 146-149.

Crouch, C.F. and Acres, S.D. (1984). Prevalence of rotavirus and coronavirus antigens in the feces of normal cows. Canadian Journal of Comparative Medicine, 48, 340-342.

Crouch, C.F., Raybould, T.J.G. and Acres, S.D. (1984). Monoclonal antibody capture enzyme-linked immunosorbent assay for detection of bovine enteric coronavirus. Journal of Clinical Microbiology, 19, 388-393.

Crouch, C.F. (1985). Vaccination against enteric rota and coronaviruses in cattle and pigs: enhancement of lactogenic immunity. Vaccine, 3, 284-291.

Crouch, C.F., Ohmann, H.B., Watts, T.C. and Babiuk, L.A. (1985). Chronic shedding of bovine enteric coronavirus antigen-antibody complexes by clinically normal cows. Journal of General Virology, 66, 1489-1500.

Cruciere, C. and Laporte, J. (1988). Sequence and analysis of bovine enteritic coronavirus (F15) genome. Annales de l'Institut Pasteur, 139, 123-138.

Czerny, C.P. and Eichhorn, W. (1989). Characterization of monoclonal and polyclonal antibodies to bovine enteric coronavirus: Establishment of an efficient ELISA for antigen detection in feces. Veterinary Microbiology, 20, 111-122.

Dauvergne, M., Laporte, J., Reynaud, G., Soulebot, J.P., Brun, A. and Espinasse, J. (1983). Vaccination of dams with a combined rotavirus-coronavirus vaccine to protect newborn calves against diarrhea. In Proceedings, Fourth International Symposium on Neonatal Diarrhea, pp. 424-434. VIDO, Saskatchewan.

Davies, H.A. and Macnaughton, M.R. (1979). Comparison of the morphology of three coronaviruses. Archives of Virology, 59, 25-33.

Dea, S., Roy, R.S. and Begin, M.E. (1979). Counterimmuno-electroosmophoresis for detection of neonatal calf diarrhea coronavirus: Methodology and comparison with electron microscopy. Journal of Clinical Microbiology, 10, 240-244.

Dea, S., Roy, R.S. and Begin, M.E. (1980a). Bovine coronavirus isolation and cultivation in continuous cell lines. American Journal of Veterinary Research, 41, 30-38.

Dea, S., Roy, R.S. and Begin, M.E. (1980b). Physicochemical and biological properties of neonatal calf diarrhea coronaviruses isolated in Quebec and comparison with the Nebraska calf coronavirus. American Journal of Veterinary Research, 41, 23-29.

Dea, S., Roy, R.S. and Elazhary, M.A.S.Y. (1982). Antigenic variations among calf diarrhea coronaviruses by immunodiffusion and counterimmunoelectrophoresis. Annales de Recherches Veterinaires, 13, 351-356.

Dea, S. and Tijssen, P. (1989a). Antigenic and polypeptide structure of turkey enteric coronaviruses as defined by monoclonal antibodies. Journal of General Virology, 70, 1725-1741.

Dea, S. and Tijssen, P. (1989b). Detection of turkey enteric coronavirus by enzyme-linked immunosorbent assay and differentiation from other coronaviruses. American Journal of Veterinary Research, 50, 226-231.

Dea, S., Garzon, S. and Tijssen, P. (1989a). Identification and location of the structural glycoproteins of a tissue culture-adapted turkey enteric coronavirus. Archives of Virology, 106, 221-237.

Dea, S., Garzon, S. and Tijssen, P. (1989b). Isolation and trypsin-enhanced propagation of turkey enteric (bluecomb) coronaviruses in a continuous human rectal adenocarcinoma cell line. American Journal of Veterinary Research, 50, 1310-1318.

Dea, S., Verbeek, A.J. and Tijssen, P. (1990). Antigenic and genomic relationships among turkey and bovine enteric coronaviruses. Journal of Virology, 64, 3112-3118.

Delmas, B., Gelfi, J. and Laude, H. (1986). Antigenic structure of transmissible gastroenteritis virus. II. Domains in the peplomer glycoprotein. Journal of General Virology, 67, 1405-1418.

Delmas, B. and Laude, H. (1990). Assembly of coronavirus spike protein into trimers and its role in epitope expression. Journal of Virology, 64, 5367-5375.

Deregt, D., Crouch, C.F., Sabara, M., Gilchrist, J.E., Babiuk, L.A. and Hudson, G.R. (1983). Preliminary studies of a bovine coronavirus (BCV) antigen responsible for neutralization. In Proceedings, Fourth International Symposium on Neonatal Diarrhoea, pp. 117-133. VIDO, Saskatchewan.

Deregt, D. and Babiuk, L.A. (1987). Monoclonal antibodies to bovine coronavirus: Characteristics and topographical mapping of neutralizing epitopes on the E2 and E3 glycoproteins. Virology, 161, 410-420.

Deregt, D., Sabara, M. and Babiuk, L.A. (1987). Structural proteins of bovine coronavirus and their intracellular processing. Journal of General Virology, 68, 2863-2877.

Deregt, D., Gifford, G.A., Ijaz, M.K., Watts, T.C., Gilchrist, J.E., Haines, D.M. and Babiuk, L.A. (1989a). Monoclonal antibodies to bovine coronavirus glycoproteins E2 and E3: Demonstration of in vivo virus-neutralizing activity. Journal of General Virology, 70, 993-998.

Deregt, D., Parker, M.D., Cox, G.C. and Babiuk, L.A. (1989b). Mapping of neutralizing epitopes to fragments of the bovine coronavirus E2 protein by proteolysis of antigen-antibody complexes. Journal of General Virology, 70, 647-658.

Doughri, A.M., Storz, J., Hajer, I. and Fernando, H.S. (1976). Morphology and morphogenesis of a coronavirus infecting intestinal epithelial cells of newborn calves. Experimental and Molecular Pathology, 25, 355-370.

Durham, P.J.K., Stevenson, B.J. and Farquharson, B.C. (1979). Rotavirus and coronavirus associated diarrhoea in domestic animals. New Zealand Veterinary Journal, 27, 30-32.

Durham, P.J.K., Hassard, L.E., Armstrong, K.R. and Naylor, J.M. (1989). Coronavirus-associated diarrhea (winter dysentery) in adult cattle. Canadian Veterinary Journal, 30, 825-827.

El-Ghorr, A.A. (1988). PhD Thesis: Investigations into the biology of bovine coronavirus and the infections it causes.

El-Ghorr, A.A., Snodgrass, D.R. and Scott, F.M.M. (1988). Evaluation of an immunogold electron microscopy technique for detecting bovine coronavirus. Journal of Virological Methods, 19, 215-224.

El-Ghorr, A.A., Snodgrass, D.R., Scott, F.M.M. and Campbell, I. (1989). A serological comparison of bovine coronavirus strains. Archives of Virology, 104, 241-248.

Espinasse, J., Viso, M., Laval, A., Savey, M., Le Layec, C.L., Blot, J.P., L'Haridon, R. and Cohen, J. (1982). Winter dysentery: A coronavirus-like agent in the faeces of beef and dairy cattle with diarrhoea. Veterinary Record, 110, 385.

Gerna, G., Cereda, P.M., Revello, M.G., Cattaneo, E., Battaglia, M. and Gerna, M.T. (1981). Antigenic and biological relationships between human coronavirus OC43 and neonatal calf diarrhoea coronavirus. Journal of General Virology, 54, 91-102.

Gershoni, J.M. and Palade, G.E. (1983). Protein blotting: Principles and applications. Analytical Biochemistry, 131, 1-15.

Gilmore, W., Fleming, J.O., Stohlman, S.A. and Weiner, L.P. (1987). Characterization of the structural proteins of the murine coronavirus strain A59 using monoclonal antibodies (42532). Proceedings of the Society for Experimental Biology and Medicine, 185, 177-186.

Goding, J.W. (1978). Use of staphylococcal protein A as an immunological reagent. Journal of Immunological Methods, 20, 241-253.

Guesdon, J.L., Ternynck, T. and Avrameas, S. (1979). The use of avidin-biotin interaction in immunoenzymatic techniques. Journal of Histochemistry and Cytochemistry, 27, 1131-1139.

Guy, J.S. and Brian, D.A. (1979). Bovine coronavirus genome. Journal of Virology, 29, 293-300.

Hajer, I. and Storz, J. (1978). Antigens of bovine coronavirus strain LY-138 and their diagnostic properties. American Journal of Veterinary Research, 39, 441-444.

Heckert, R.A., Saif, L.J. and Myers, G.W. (1989). Development of protein A-gold immunoelectron microscopy for detection of bovine coronavirus in calves: Comparison with ELISA and direct immunofluorescence of nasal epithelial cells. Veterinary Microbiology, 19, 217-231.

Heckert, R.A., Saif, L.J., Hoblet, K.H. and Agnes, A.G. (1990). A longitudinal study of bovine coronavirus enteric and respiratory infections in dairy calves in two herds in Ohio. Veterinary Microbiology, 22, 187-201.

Herring, A.J., Inglis, N.F., Ojeh, C.K., Snodgrass, D.R. and Menzies, J.D. (1982). Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. Journal of Clinical Microbiology, 16, 473-477.

Herring, A.J. and Sharp, J.M. (1984). Protein blotting: The basic method and its role in viral diagnosis. In Recent Advances in Virus Research, pp. 115-124. Edited by M.S. McNulty and J.B. McFerran. Martinus Nijhoff Publishers.

Hirano, N., Sada, Y., Tuchiya, K., Ono, K. and Murakami, T. (1985). Plaque assay of bovine coronavirus in BEK-1 cells. Japanese Journal of Veterinary Science, 47, 679-681.

Hofmann, M.A., Sethna, P.B. and Brian, D.A. (1990). Bovine coronavirus mRNA replication continues throughout persistent infection in cell culture. Journal of Virology, 64, 4108-4114.

Hogue, B.G., King, B. and Brian, D.A. (1984). Antigenic relationships among proteins of bovine coronavirus, human respiratory coronavirus OC43, and mouse hepatitis coronavirus A59. Journal of Virology, 51, 384-388.

Hogue, B.G., Kienzle, T.E. and Brian, D.A. (1989). Synthesis and processing of the bovine enteric coronavirus haemagglutinin protein. Journal of General Virology, 70, 345-352.

Holmes, K.V., Doller, E.W. and Behnke, J.N. (1981). Analysis of the functions of coronavirus glycoproteins by differential inhibition of synthesis with tunicamycin. Advances in Experimental Medicine and Biology, 142, 133-142.

Holmes, K.V. (1985). Replication of coronaviruses. In Virology, pp. 1331-1343. Edited by B.N. Fields et al. New York: Raven Press.

Holmes, K.V., Welsh, R.M. and Haspel, M.V. (1986). Natural cytotoxicity against mouse hepatitis virus-infected target cells. Journal of Immunology, 136, 1446-1453.

Holmes, K.V. (1990). Coronaviridae and their replication. In Virology, pp. 841-856. Edited by B.N. Fields et al. New York: Raven Press.

Horner, G.W., Hunter, R. and Kirkbride, C.A. (1975). Coronavirus-like agent present in faeces of cows with diarrhoea. New Zealand Veterinary Journal, 23, 98.

Horzinek, M.C., Weiss, M. and Ederveen, J. (1984). Berne virus is not 'coronavirus-like'. Journal of General Virology, 65, 645-649.

Inaba, Y., Sato, K., Kurogi, H., Takahashi, E., Ito, Y., Omori, T., Goto, Y. and Matumoto, M. (1976). Replication of bovine coronavirus in cell line BEK-1 culture. Archives of Virology, 50, 339-342.

Iorio, R.M. and Bratt, M.A. (1984). Neutralization of Newcastle disease virus by monoclonal antibodies to the haemagglutinin-neuraminidase glycoprotein: Requirement for antibodies to four sites for complete neutralization. Journal of Virology, 51, 445-451.

Kapil, S., Trent, A.M. and Goyal, S.M. (1990). Excretion and persistence of bovine coronavirus in neonatal calves. Archives of Virology, 115, 127-132.

Karber, G. (1931). Beitrag zur kollektiven behandlung pharmakologischer reihenversuche. Archiv fur Experimentelle Pathologie und Pharmakologie, 162, 480-483.

Kaye, H.S. and Dowdle, W.R. (1969). Some characteristics of hemagglutination of certain strains of "IBV-like" virus. Journal of Infectious Diseases, 120, 576-581.

Kaye, H.S., Yarbrough, W.B. and Reed, C.J. (1975). Calf diarrhoea coronavirus. Lancet ii, 509.

Keck, J.G., Hogue, B.G., Brian, D.A. and Lai, M.M.C. (1988). Temporal regulation of bovine coronavirus RNA synthesis. Virus Research, 9, 343-356.

Kendall, C., Ionescu-Matiu, I. and Dreesman, G.R. (1983). Utilization of the biotin/avidin system to amplify the sensitivity of the enzyme-linked immunosorbent assay (ELISA). Journal of Immunological Methods, 56, 329-339.

Kienzle, T.E., Abraham, S., Hogue, B.G. and Brian, D.A. (1990). Structure and orientation of expressed bovine coronavirus hemagglutinin-esterase protein. Journal of Virology, 64, 1834-1838.

King, B. and Brian, D.A. (1982). Bovine coronavirus structural proteins. Journal of Virology, 42, 700-707.

King, B., Potts, B.J. and Brian, D.A. (1985). Bovine coronavirus hemagglutinin protein. Virus Research, 2, 53-59.

Kohler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 256, 495-497.

Koolen, M.J.M., Osterhaus, A.D.M.E., Siebelink, K.H.J., Horzinek, M.C. and Zeijst, B.A.M. (1984). Monoclonal antibodies to the three classes of mouse hepatitis virus strain A59 proteins. In Molecular Biology and Pathogenesis of Coronaviruses, pp. 115-116. Edited by P.J.M. Rottier et al. New York and London: Plenum Press.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

Lai, M.M.C. (1990). Coronavirus: Organization, replication and expression of genome. Annual Review of Microbiology, 44, 303-333.

Langpap, T.J., Bergeland, M.E. and Reed, D.E. (1979). Coronaviral enteritis of young calves: Virologic and pathologic findings in naturally occurring infections. American Journal of Veterinary Research, 40, 1476-1478.

Laporte, J., L'Haridon, R. and Bobulesco, P. (1979). In vitro culture of bovine enteric coronavirus (BEC). Les Colloques de l'INSERM, 90, 99-102.

Laporte, J., Bobulesco, P. and Rossi, F. (1980). Une lignee cellulaire particulierement sensible a la replication du coronavirus enteritique bovin: les cellules HRT18. Communiques Recherches de L'Academie des Sciences, 290, 623-626.

Laporte, J. and Bobulesco, P. (1981a). Growth of human and canine enteritic coronaviruses in a highly susceptible cell line: HRT18. In Perspectives in Virology, XI, pp. 189-193. New York: Alan R. Liss, Inc.

Laporte, J. and Bobulesco, P. (1981b). Polypeptide structure of bovine enteritic coronavirus: comparison between a wild strain purified from feces and a HRT18 cell adapted strain. Advances in Experimental Medicine and Biology, 142, 181-184.

Lapps, W. and Brian, D.A. (1985). Oligonucleotide fingerprints of antigenically related bovine coronavirus and human coronavirus OC43. Archives of Virology, 86, 101-108.

Lapps, W., Hogue, B.G. and Brian, D.A. (1987). Sequence analysis of the bovine coronavirus nucleocapsid and matrix protein genes. Virology, 157, 47-57.

Laude, H., Chapsal, J.M., Gelfi, J., Labiau, S. and Grosclaude, J. (1986). Antigenic structure of transmissible gastroenteritis virus. I. Properties of monoclonal antibodies directed against virion proteins. Journal of General Virology, 67, 119-130.

Laver, W.G., Air, G.M., Webster, R.G. and Smith-Gill, S.J. (1990). Epitopes on protein antigens: Misconceptions and realities. Cell, 61, 553-556.

Lecomte, J., Cainelli-Gebara, V., Mercier, G., Mansour, S., Talbot, P.J., Lussier, G. and Oth, D. (1987). Protection from mouse hepatitis virus type 3-induced acute disease by an anti-nucleoprotein monoclonal antibody. Archives of Virology, 97, 123-130.

Lewis, L.D. and Phillips, R.W. (1978). Pathophysiologic changes due to coronavirus-induced diarrhea in the calf. Journal of the American Veterinary Medical Association, 173, 636-642.

Luytjes, W., Bredenbeek, P.J., Noten, A.F.H., Horzinek, M.C. and Spaan, W.J.M. (1988). Sequence of mouse hepatitis virus A59 mRNA2: Indications for RNA recombination between coronaviruses and influenza C virus. Virology, 166, 415-422.

Marsolais, G., Assaf, R., Montpetit, C. and Marois, P. (1978). Diagnosis of viral agents associated with neonatal calf diarrhoea. Canadian Journal of Comparative Medicine, 42, 168-171.

McIntosh, K. (1974). Coronaviruses: A comparative review. Current topics in Microbiology and Immunology, 63, 85-129.

McNulty, M.S., Bryson, D.G., Allan, G.M. and Logan, E.F. (1984). Coronavirus infection of the bovine respiratory tract. Veterinary Microbiology, 9, 425-434.

Mebus, C.A., White, R.G., Stair, E.L., Rhodes, M.B. and Twiehaus, M.J. (1972). Neonatal calf diarrhea: Results of a field trial using a reo-like virus vaccine. Veterinary Medicine/ Small Animal Clinician, 67, 173-178.

Mebus, C.A., Stair, E.L., Rhodes, M.B. and Twiehaus, M.J. (1973a). Neonatal calf diarrhea: Propagation, attenuation, and characteristics of a coronavirus-like agent. American Journal of Veterinary Research, 34, 145-150.

Mebus, C.A., Stair, E.L., Rhodes, M.B. and Twiehaus, M.J. (1973b). Pathology of neonatal calf diarrhea induced by a coronavirus-like agent. Veterinary Pathology, 10, 45-64.

Mebus, C.A., Newman, L.E. and Stair, E.L. (1975). Scanning electron, light and immunofluorescent microscopy of intestine of gnotobiotic calf infected with calf diarrheal coronavirus. American Journal of Veterinary Research, 36, 1719-1725.

Moon, H.W., McClurkin, A.W., Isaacson, R.E., Pohlenz, J., Skartvedt, S.M., Gillette, K.G. and Baetz, A.L. (1978). Pathogenic relationships of rotavirus, Escherichia coli, and other agents in mixed infections in calves. Journal of the American Veterinary Medical Association, 173, 577-583.

Mostl, K. and Burki, F. (1989). Bovine coronavirus is also a cause of respiratory disease in calves. In Proceedings, Eleventh International Symposium of the World Association of Veterinary Microbiologists, Immunologists and Specialists in Infectious Diseases, pp. 191. Perugia, Italy.

Myers, L.L. and Snodgrass, D.R. (1982). Colostral and milk antibody titers in cows vaccinated with a modified live-rotavirus-coronavirus vaccine. Journal of the American Veterinary Medical Association, 181, 486-488.

Parker, M.D., Cox, G.J., Deregt, D., Fitzpatrick, D.R. and Babiuk, L.A. (1989). Cloning and in vitro expression of the gene for the E3 haemagglutinin glycoprotein of bovine coronavirus. Journal of General Virology, 70, 155-164.

Parker, M.D., Yoo, D., Cox, G.J. and Babiuk, L.A. (1990). Primary structure of the S peplomer gene of bovine coronavirus and surface expression in insect cells. Journal of General Virology, 71, 263-270.

Patel, J.R., Davies, H.A., Edington, N., Laporte, J. and MacNaughton, M.R. (1982). Infection of a calf with the enteric coronavirus strain Paris. Archives of Virology, 73, 319-327.

Payne, H.R. and Storz, J. (1988). Analysis of cell fusion induced by bovine coronavirus infection. Archives of Virology, 103, 27-33.

Payne, H.R., Storz, J. and Henk, W.G. (1990a). Initial events in bovine coronavirus infection: analysis through immunogold probes and lysosomotropic inhibitors. Archives of Virology, 114, 175-189.

Payne, H.R., Storz, J. and Henk, W.G. (1990b). Bovine coronavirus antigen in the host cell plasmalemma. Experimental and Molecular Pathology, 53, 152-159.

Pedersen, N.C., Ward, J. and Mengeling, W.L. (1978). Antigenic relationship of the feline infectious peritonitis virus to coronaviruses of other species. Archives of Virology, 58, 45-53.

Peiris, J.S.M. and Porterfield, J.S. (1981). Antibody-dependent enhancement of plaque formation on cell lines of macrophage origin - A sensitive assay for antiviral antibody. Journal of General Virology, 57, 119-125.

Pensaert, M. and Callebaut, P. (1978). The coronaviruses: Clinical and structural aspects with some practical implications. Annales de Medecine Veterinaire, 122, 301-322.

Posthumus, W.P.A., Lenstra, J.A., Schaaper, W.M.M., Van Nieuwstadt, A.P., Enjuanes, L. and Meloen, R.H. (1990). Analysis and simulation of a neutralizing epitope of transmissible gastroenteritis virus. Journal of Virology, 64, 3304-3309.

Radostits, O.M. and Acres, S.D. (1980). The prevention and control of epidemics of acute undifferentiated diarrhea of beef calves in western Canada. Canadian Veterinary Journal, 21, 243-249.

Ramig, R.F. (1988). The effects of host age, virus dose, and virus strain on heterologous rotavirus infection of suckling mice. Microbial Pathogenesis, 4, 189-202.

Reynolds, D.J., Chasey, D., Scott, A.C. and Bridger, J.C. (1984). Evaluation of ELISA and electron microscopy for the detection of coronavirus and rotavirus in bovine faeces. Veterinary Record, 114, 397-401.

Reynolds, D.J., Debney, T.G., Hall, G.A., Thomas, L.H. and Parsons, K.R. (1985). Studies on the relationship between coronaviruses from the intestinal and respiratory tracts of calves. Archives of Virology, 85, 71-83.

Reynolds, D.J., Morgan, J.H., Chanter, N., Jones, P.W., Bridger, J.C., Debney, T.G. and Bunch, K.J. (1986). Microbiology of calf diarrhoea in southern Britain. Veterinary Record, 119, 34-39.

Ritchie, A.E., Deshmukh, D.R., Larsen, C.T. and Pomeroy, B.S. (1973). Electron microscopy of coronavirus-like particles characteristic of turkey bluecomb disease. Avian Disease, 17, 546-559.

Rodak, L., Babiuk, L.A. and Acres, S.D. (1982). Detection by radioimmunoassay and enzyme-linked immunosorbent assay of coronavirus antibodies in bovine serum and lacteal secretions. Journal of Clinical Microbiology, 16, 34-40.

Roseto, A., Bobulesco, P., Laporte, J., Escaig, J., Gaches, D. and Peries, J. (1982). Bovine enteric coronavirus structure as studied by a freeze-drying technique. Journal of General Virology, 63, 241-245.

Saif, L.J. (1985). Passive immunity to coronavirus and rotavirus infections in swine and cattle: Enhancement by maternal vaccination. In Infectious diarrhoea in the Young, pp. 456-467. Edited by S. Tzipori et al. Elsevier Science Publishers, B.V. (Biomedical Division).

Saif, L.J., Redman, D.R., Moorhead, P.D. and Theil, K.W. (1986). Experimentally induced coronavirus infections in calves: Viral replication in the respiratory and intestinal tracts. American Journal of Veterinary Research, 47, 1426-1432.

Saif, L.J. (1987). Development of nasal, fecal and serum isotype-specific antibodies in calves challenged with bovine coronavirus or rotavirus. Veterinary Immunology and Immunopathology, 17, 425-439.

Saif, L.J., Redman, D.R., Brock, K.V., Kohler, E.M. and Heckert, R.A. (1988). Winter dysentery in adult dairy cattle: Detection of coronavirus in the faeces. Veterinary Record, 123, 300-301.

Saif, L.J. (1990). A review of evidence implicating bovine coronavirus in the etiology of winter dysentery in cows: An enigma resolved? Cornell Veterinarian, 80, 303-311.

Sato, K., Inaba, Y., Kurogi, H., Takahashi, E., Satoda, K., Omori, T. and Matumoto, M. (1977). Hemagglutination by calf diarrhea coronavirus. Veterinary Microbiology, 2, 83-87.

Sato, K., Inaba, Y. and Matumoto, M. (1980). Serological relation between calf diarrhea coronavirus and hemagglutinating encephalomyelitis virus. Archives of Virology, 66, 157-159.

Sato, K., Inaba, Y., Tokuhisa, S., Miura, Y., Kaneko, N., Asagi, M. and Matumoto, M. (1984). Detection of bovine coronavirus in feces by reversed passive hemagglutination. Archives of Virology, 80, 23-31.

Schalk, A.F. and Hawn, M.C. (1931). An apparently new respiratory disease of baby chicks. Journal of the American Veterinary Medical Association, 78, 413-422.

Schultze, B., Gross, H.J., Brossmer, R., Klenk, H.D. and Herrler, G. (1990). Hemagglutinating encephalomyelitis virus attaches to N-acetyl-9-0-acetylneuraminic acid-containing receptors on erythrocytes: comparison with bovine coronavirus and influenza C virus. Virus Research, 16, 185-194.

Schultze, B., Wahn, K., Klenk, H.D. and Herrler, G. (1991). Isolated HE-protein from hemagglutinating encephalomyelitis virus and bovine coronavirus has receptor-destroying and receptor-binding activity. Virology, 180, 221-228.

Sharpee, R.L., Mebus, C.A. and Bass, E.P. (1976). Characterization of a calf diarrheal coronavirus. American Journal of Veterinary Research, 37, 1031-1041.

Shockley, L.J., Kapke, P.A., Lapps, W., Brian, D.A., Potgieter, L.N.D. and Woods, R. (1987). Diagnosis of porcine and bovine enteric coronavirus infections using cloned cDNA probes. Journal of Clinical Microbiology, 25, 1591-1596.

Siddell, S.G., Barthel, A. and Ter Meulen, V. (1981). Coronavirus JHM: a virion-associated protein kinase. Journal of General Virology, 52, 235-243.

Siddell, S., Wege, H. and Ter Meulen, V. (1982). The structure and replication of coronaviruses. Current Topics in Microbiology and Immunology, 99, 131-163.

Siddell, S., Wege, H. and Ter Meulen, V. (1983). The biology of coronaviruses. Journal of General Virology, 64, 761-776.

Siddell, S. (1987). The organization and expression of coronavirus genomes. In The Molecular Biology of the Positive Strand RNA Viruses, pp. 117-127. Academic Press Inc. (London) Ltd.

Simkins, R.A., Saif, L.J. and Weilnau, P.A. (1989). Epitope mapping and the detection of transmissible gastroenteritis viral proteins in cell culture using biotinylated monoclonal antibodies in a fixed-cell ELISA. Archives of Virology, 107, 179-190.

Singh, D.K., Singh, N.P. and Singh, G.K. (1985). Pneumoenteric syndrome in bovine neonates by bovine coronavirus. Indian Journal of Veterinary Medicine, 5, 55-57.

Snijder, E.J., Boon, J.A., Bredenbeek, P.J., Horzinek, M.C., Rijnbrand, R. and Spaan, W.J.M. (1990). The carboxyl-terminal part of the putative Berne virus polymerase is expressed by ribosomal frameshifting and contains sequence motifs which indicate that toro- and coronaviruses are evolutionarily related. Nucleic Acids Research, 18, 4535-4542.

Snijder, E.J., Den Boon, J.A., Horzinek, M.C. and Spaan, W.J.M. (1991). Comparison of the genome organization of toro- and coronaviruses: Evidence for two non-homologous RNA recombination events during Berne virus evolution. Virology, 180, 448-452.

Snodgrass, D.R., Fahey, K.J., Wells, P.W., Campbell, I. and Whitelaw, A. (1980). Passive immunity in calf rotavirus infections: Maternal vaccination increases and prolongs immunoglobulin G1 antibody secretion in milk. Infection and Immunity, 28, 344-349.

Snodgrass, D.R., Stewart, J., Taylor, J., Krautil, F.L. and Smith, M.L. (1982). Diarrhoea in dairy calves reduced by feeding colostrum from cows vaccinated with rotavirus. Research in Veterinary Science, 32, 70-73.

Snodgrass, D.R., Terzolo, H.R., Sherwood, D., Campbell, I., Menzies, J.D. and Synge, B.A. (1986). Aetiology of diarrhoea in young calves. Veterinary Record, 119, 31-34.

Spaan, W., Cavanagh, D. and Horzinek, M.C. (1988). Coronaviruses: structure and genome expression. Journal of General Virology, 69, 2939-2952.

Spaan, W., Cavanagh, D. and Horzinek, M.C. (1990). Coronaviruses. In Immunochemistry of viruses, II. The basis for serodiagnosis and vaccines, pp. 359-379. Edited by M.H.V. van Regenmortel and A.R. Neurath. Elsevier Science Publishers B.V. (Biomedical Division).

St. Cyr-Coats, K.S., Storz, J., Hussain, K.A. and Schnorr, K.L. (1988). Structural proteins of bovine coronavirus strain L9: effects of the host cell and trypsin treatment. Archives of Virology, 103, 35-45.

Stair, E.L., Rhodes, M.B., White, R.G. and Mebus, C.A. (1972). Neonatal calf diarrhea: Purification and electron microscopy of a coronavirus-like agent. American Journal of Veterinary Research, 33, 1147-1156.

Storz, J. and Rott, R. (1981). Reactivity of antibodies in human serum with antigens of an enteropathogenic bovine coronavirus. Medical Microbiology and Immunology, 169, 169-178.

Storz, J., Kaluza, G., Niemann, H. and Rott, R. (1981a). On enteropathogenic bovine coronavirus. In Biochemistry and Biology of Coronaviruses, pp. 171-179. Edited by V. ter Meulen et al. New York and London: Plenum Press.

Storz, J., Rott, R. and Kuluza, G. (1981b). Enhancement of plaque formation and cell fusion of an enteropathogenic coronavirus by trypsin treatment. Infection and Immunity, 31, 1214-1222.

Storz, J., Herrler, G., Snodgrass, D.R., Hussain, K.A., Zhang, X.M., Clark, M.A. and Rott, R. (1991). Monoclonal antibodies differentiate between the hemagglutinating and the receptor-destroying activities of bovine coronavirus. (personal communication).

Stott, E.J., Thomas, L.H., Bridger, J.C. and Jebbett, N.J. (1976). Replication of a bovine coronavirus in organ cultures of foetal trachea. Veterinary Microbiology, 1, 65-69.

Sturman, L.S. (1977). Characterization of a coronavirus. 1. Structural proteins: effects of preparative conditions on the migration of protein in polyacrylamide gels. Virology, 77, 637-649.

Sturman, L.S., Holmes, K.V. and Behnke, J. (1980). Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. Journal of Virology, 33, 449-462.

Sturman, L.S. and Holmes, K.V. (1983). The molecular biology of coronaviruses. Advances in Virus Research, 28, 35-112.

Sturman, L.S., Ricard, C.S. and Holmes, K.V. (1985). Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: activation of cell-fusing activity of virions by trypsin and separation of two different 90K cleavage fragments. Journal of Virology, 56, 904-911.

Taguchi, F. and Fleming, J.O. (1989). Comparison of six different murine coronavirus JHM variants by monoclonal antibodies against the E2 glycoprotein. Virology, 169, 233-235.

Takahashi, E., Inaba, Y., Sato, K., Ito, Y., Kurogi, H., Akashi, H., Satoda, K. and Omori, T. (1980). Epizootic diarrhoea of adult cattle associated with a coronavirus-like agent. Veterinary Microbiology, 5, 151-154.

Takahashi, E., Akashi, H. and Inaba, Y. (1983). Bovine epizootic diarrhea resembling winter dysentery caused by bovine coronavirus. JARQ 17, 37-42.

Talbot, P.J., Salmi, A.A., Knobler, R.L. and Buchmeier, M.J. (1984). Topographical mapping of epitopes on the glycoproteins of murine hepatitis virus-4 (strain JHM): Correlation with biological activities. Virology, 132, 250-260.

Tasheva, B. and Dessev, G. (1983). Artifacts in sodium dodecyl sulfate-polyacrylamide gel electrophoresis due to 2-mercaptoethanol. Analytical Biochemistry, 129, 98-102.

Tektoff, J., Dauvergne, M., Durafour, M. and Soulebot, J.P. (1983a). Propagation of bovine coronavirus on vero cell line: Electron microscopic studies. Developments in Biological Standardization, 53, 299-310.

Tektoff, J., Dauvergne, M., Durafour, M. and Soulebot, J.P. (1983b). Propagation of bovine enteritic coronavirus in three cell systems: Electron microscopic studies. In Proceedings, Fourth International Symposium on Neonatal Diarrhoea, pp. 134-153. VIDO, Saskatchewan.

Thomas, L.H., Gourlay, R.N., Stott, E.J., Howard, C.J. and Bridger, J.C. (1982). A search for new microorganisms in calf pneumonia by the inoculation of gnotobiotic calves. Research in Veterinary Science, 33, 170-182.

Thurber, E.T., Bass, E.P. and Beckenhauer, W.H. (1977). Field trial evaluation of a reo-coronavirus calf diarrhea vaccine. Canadian Journal of Comparative Medicine, 41, 131-136.

Tompkins, W.A.F., Watrach, A.M., Schmale, J.D., Schultz, R.M. and Harris, J.A. (1974). Cultural and antigenic properties of newly established cell strains derived from adenocarcinomas of the human colon and rectum. Journal of the National Cancer Institute, 52, 1101-1110.

Toth, T.E. (1982). Trypsin-enhanced replication of neonatal calf diarrhea coronavirus in bovine embryonic lung cells. American Journal of Veterinary Research, 43, 967-972.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceedings of the National Academy of Sciences, U.S.A., 76, 4350-4354.

Towbin, H. and Gordon, J. (1984). Immunoblotting and dot immunobinding - Current status and outlook. Journal of Immunological Methods, 72, 313-340.

Tyrrell, D.A. and Bynoe, M.L. (1965). Cultivation of a novel type of common-cold virus in organ cultures. British Medical Journal, 5448, 1467-1470.

Tyrrell, D.A.J., Almeida, J.D., Cunningham, C.H., Dowdle, W.R., Hofstad, M.S., McIntosh, K., Tajima, M., Zakstelskaya, L.Y., Easterday, B.C., Kapikian, A. and Bingham, R.W. (1975). Coronaviridae. Intervirology, 5, 76-82.

Underdahl, N.R., Mebus, C.A., Stair, E.L., Rhodes, M.B., McGill, L.D. and Twiehaus, M.J. (1974). Isolation of transmissible gastroenteritis virus from lungs of market-weight swine. American Journal of Veterinary Research, 35, 1209-1216.

Utter, G., Mufson, M.A. and Norrby, E. (1986). Detection of antigen in immune precipitates by silver staining of SDS-polyacrylamide gels. Journal of Virological Methods, 13, 35-42.

Van Balken, J.A.M., De Leeuw, P.W., Ellens, D.J. and Straver, P.J. (1978). Detection of coronavirus in calf faeces with a haemadsorption-elution-haemagglutination assay (HEHA). Veterinary Microbiology, 3, 205-211.

Van Kruiningen, H.J., Khairallah, L.H., Sasseville, V.G., Wyand, M.S. and Post, J.E. (1987). Calfhood coronavirus enterocolitis: A clue to the etiology of winter dysentery. Veterinary Pathology, 24, 564-567.

Vautherot, J.F. (1981). Plaque assay for titration of bovine enteric coronavirus. Journal of General Virology, 56, 451-455.

Vautherot, J.F. and Laporte, J. (1983). Utilization of monoclonal antibodies for antigenic characterization of coronaviruses. Annales de Recherches Veterinaires, 14, 437-444.

Vautherot, J.F., Laporte, J., Madelaine, M.F., Bobulesco, P. and Roseto, A. (1984). Antigenic and polypeptide structure of bovine enteric coronavirus as defined by monoclonal antibodies. In Molecular Biology and Pathogenesis of Coronaviruses, pp. 117-132. Edited by P.J.M. Rottier et al. New York: Plenum Press.

Verbeek, A. and Tijssen, P. (1988). Biotinylated and radioactive cDNA probes in the detection by hybridization of bovine enteric coronavirus. Molecular and Cellular Probes, 2, 209-223.

Verbeek, A., Dea, S. and Tijssen, P. (1990). Detection of bovine enteric coronavirus in clinical specimens by hybridization with cDNA probes. Molecular and Cellular Probes, 4, 107-120.

Verbeek, A. and Tijssen, P. (1990). Polymerase chain reaction for probe synthesis and for direct amplification in detection of bovine coronavirus. Journal of Virological Methods, 29, 243-256.

de Visser, N.A.P.C., Breukink, H.J., van Zijderveld, F.G. and de Leeuw, P.W. (1987). Enteric infections in veal calves: a longitudinal study on four veal calf units. Veterinary Quarterly, 9, 289-296.

Vlasak, R., Luytjes, W., Leider, J., Spaan, W. and Palese, P. (1988a). The E3 protein of bovine coronavirus is a receptor-destroying enzyme with acetylesterase activity. Journal of Virology, 62, 4686-4690.

Vlasak, R., Luytjes, W., Spaan, W. and Palese, P. (1988b). Human and bovine coronaviruses recognize sialic acid-containing receptors similar to those of influenza C viruses. Proceedings of the National Academy of Science, USA, 85, 4526-4529.

Waltner-Toews, D., Martin, S.W., Meek, A.H. and McMillan, I. (1983). A field trial to test the efficacy of a combined rotavirus-coronavirus/E. coli vaccine in dairy cattle. In Proceedings, Fourth International Symposium on Neonatal Diarrhea, pp. 456-483. VIDO, Saskatchewan.

Wege, H., Siddell, S. and Ter Meulen, V. (1982). The biology and pathogenesis of coronaviruses. Current Topics in Microbiology and Immunology, 99, 165-200.

Wege, H., Dorries, R. and Wege, H. (1984). Hybridoma antibodies to the murine coronavirus JHM: Characterization of epitopes on the peplomer protein (E2). Journal of General Virology, 65, 1931-1942.

Wege, H., Winter, J. and Meyermann, R. (1988). The peplomer protein E2 of coronavirus JHM as a determinant of neurovirulence: definition of critical epitopes by variant analysis. Journal of General Virology, 69, 87-98.

Weiss, M. and Horzinek, M.C. (1987). The proposed family Toroviridae: Agents of enteric infections. Archives of Virology, 92, 1-15.

Welch, S.K.W. and Saif, L.J. (1988). Monoclonal antibodies to a virulent strain of transmissible gastroenteritis virus: comparison of reactivity with virulent and attenuated virus. Archives of Virology, 101, 221-235.

Woode, G.N. and Bridger, J.C. (1975). Viral enteritis of calves. Veterinary Record, 96, 85-88.

Woode, G.N., Bridger, J.C. and Meyling, A. (1978). Significance of bovine coronavirus infection. Veterinary Record, 102, 15-16.

Woode, G.N., Reed, D.E., Runnels, P.L., Herrig, M.A. and Hill, H.T. (1982). Studies with an unclassified virus isolated from diarrhoeic calves. Veterinary Microbiology, 7, 221-240.

Yewdell, J.W. and Gerhard, W. (1981). Antigenic characterization of viruses by monoclonal antibodies. Annual Review of Microbiology, 35, 185-206.

Yoo, D., Parker, M.D. and Babiuk, L.A. (1991). The S2 subunit of the spike glycoprotein of bovine coronavirus mediates membrane fusion in insect cells. Virology, 180, 395-399.

Zygraich, N., Georges, A.M. and Vascoboinic, E. (1975). Etiologie des diarrhees neonatales du veau. Resultats d'une enquete serologique relative aux virus reo-like et corona dans la population bovine belge. Annales de Medecine Veterinaire, 119, 105-113.